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SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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*No. 1. SIMON FLEXNER, J. W. JOBLING, and MAUD L. MENTEN. Experimental studies on tumor. Plates 1–28. (Issued June 30, 1910.) Price, $1.00.

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SURFACE TENSION OF SERUM.

XV. The Thickness of the Monolayer of Rabbit Plasma.

By P. LECOMTE du NOÜY, Sc.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, April 5, 1927.)

In the preceding papers of this series,[1] it has been shown that a well defined minimum value of the static surface tension of fresh, clear serum could be observed at a given high dilution, and the conclusion was reached that under given conditions serum and serum solutions behave as true solutions. In order to explain the presence of this minimum, —or of these minima, as in most cases more than one can be observed, —it was necessary to resort to the hypothesis that all the constituents of the serum acted as though they were bound together in the shape of a huge molecule and that at some critical concentrations, the organization of these molecules in the surface layer decreased the value of the tension measured by the du Noüy tensiometer. The similarity of this phenomenon with that observed in the case of sodium oleate,[2] egg albumin,[3] and recently digitonin,[4] the important decrease in the rate of evaporation at the same critical concentration,[5] and the displacement of the minimum when the surface of adsorption was changed,[6] seemed to bear out this hypothesis satisfactorily.

The question then arose as to whether solutions of plasma would behave in the same way, namely, as large molecules capable of organizing themselves at a given concentration, or whether they would behave as though molecules of another substance but of approximately the

[1] du Noüy, P. L., Surface tension of serum, Papers I–X, *J. Exp. Med.*, 1922, xxxv, to 1924, xl; Paper XI, *J. Gen Physiol.*, 1924, vi, 625; Papers XII–XIV, *J. Exp. Med.*, 1925, xli to xlii.

[2] du Noüy, P. L., *Phil. Mag.*, 1924, xlviii, 264, 664.

[3] du Noüy, P. L., *J. Biol. Chem.*, 1925, lxiv, 595.

[4] Unpublished experiments.

[5] du Noüy, P. L., *J. Exp. Med.*, 1924, xxxix, 717.

[6] du Noüy, P. L., *J. Exp. Med.*, 1924, xl, 133.

same molecular weight were added to the serum. In other words, when fibrinogen is present in the serum, does it exist separately, or is it part of the serum molecule, which would then be a "plasma molecule"?

If the fibrinogen is supposed to exist as a separate substance in solution (plasma = serum molecules + fibrinogen molecules), then the concentration at which the principal minimum of surface tension occurs will not be changed as the space occupied in the horizontal plane by the "serum molecules" will be the same. By diluting serum or plasma 10,500 times, a minimum should be expected in both cases, although the concentration of the latter in proteins would be greater, and consequently also the thickness of the adsorbed layer. The same would happen if the fibrinogen were bound at one end of the serum molecule, without involving any change in structure. Therefore, in case no shift should be observed, no definite answer could be given as to whether the fibrinogen exists separately or whether it is merely an easily detachable group fixed at one end of the serum molecule.

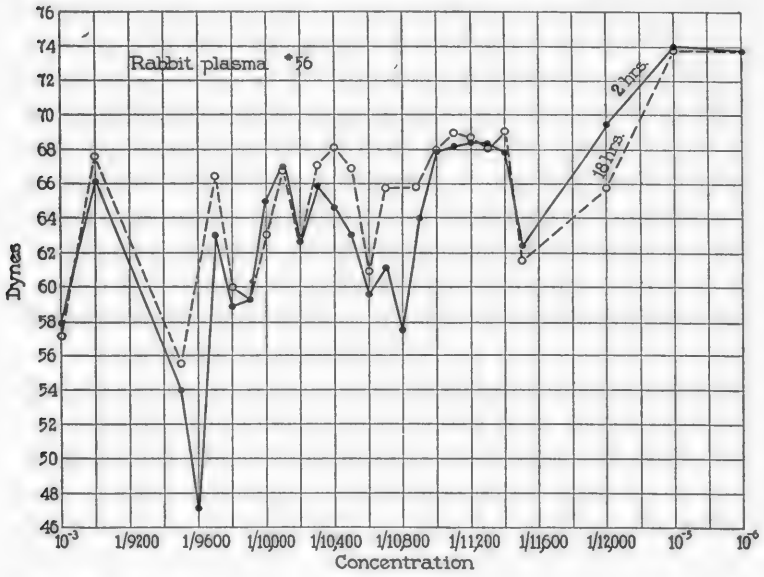
But if the fibrinogen, instead of being merely additive, were actually part of the molecule, the whole structure and symmetry of the assumed "plasma molecule" might be different from that of the "serum molecule," and consequently, the space occupied in the horizontal plane by these two molecules might also differ. If it is assumed that the "plasma molecule" can orient itself in a monolayer as does the "serum molecule," a shift in the place of the minimum is to be expected. Therefore, should a minimum be observed with plasma solutions, and should this minimum occur at the same concentration as it does with serum, it would indicate that the orientation and dimensions of the serum molecules in the horizontal plane are unchanged. In this case, no answer could be given as to the possible link between fibrinogen and serum; on the contrary, should the principal minimum be shifted toward higher or lower concentrations, one could logically assume that the molecules present in the solution are different in size and shape from those of the serum, but similarly capable of organizing as polarized units.

EXPERIMENTAL AND RESULTS.

The blood was collected and centrifuged immediately, and the plasma was diluted as soon as possible in saline solution. In a few

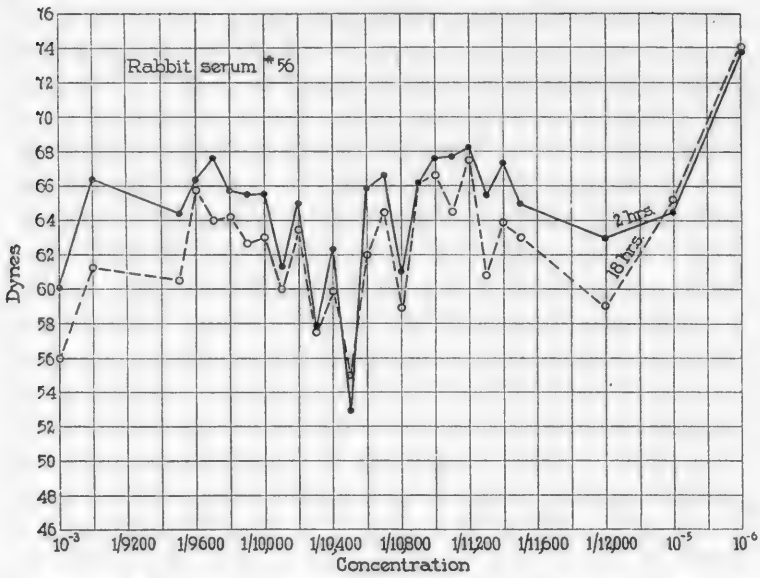
cases, coagulation occurred at the concentration 1:100, but never at 1:1000. When dilutions were made rapidly, no difficulty was encountered. The experimental part of this work was carried out by Mr. J. Zwick. (Text-figs. 1 and 2.)

The results of twenty-two series of measurements are not as sharply defined as one might have hoped. However, when the frequency of occurrence of the minima at a given concentration is taken into consideration (Text-fig. 3), it is clear that minima are observed in the case of

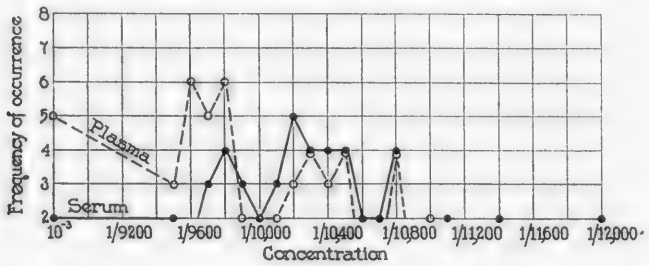


TEXT-FIG. 1. Experiment 1. Static values of diluted rabbit plasma, as a function of the concentration. The two curves give the results of two series of measurements, one after 2 hours, the other after 18 hours.

plasma and that the most important of them are shifted with respect to those of the serum. Text-fig. 4 expresses the mean value of the twenty-two curves. If the minima which only occurred twice or less are omitted as due to experimental errors, it is obvious from Text-fig. 3 that out of thirty-eight minima observed in the eleven series of experiments with plasma, twenty or 53 per cent occurred at concentrations between 1:9500 and 1:9800. Out of the thirty-four minima found for



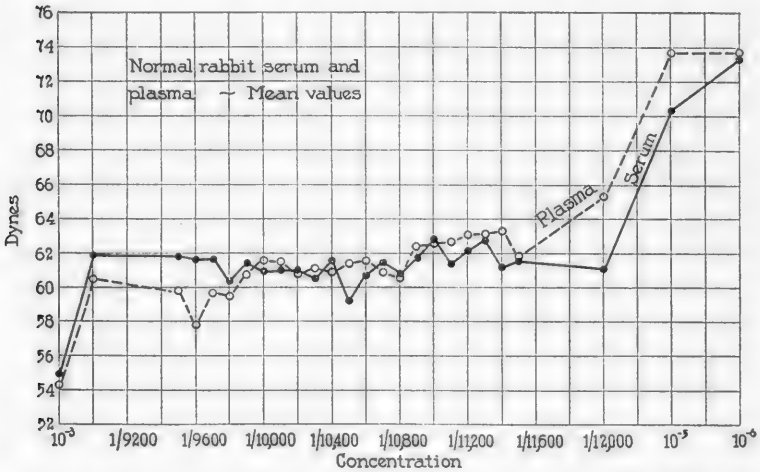
TEXT-FIG. 2. Experiment 2. Static values of diluted rabbit serum, as a function of the concentration. The two curves give the results of two series of measurements, one after 2 hours, the other after 18 hours.



TEXT-FIG. 3. Frequency of occurrence of the minima of static surface tension in plasma and serum (eleven experiments for each).

serum under the same conditions, seventeen or 50 per cent occurred at concentrations between 1:10,200 and 1:10,500. Although the minima 1:9600 for plasma and 1:10,500 for serum are quantitatively more important, as is seen from Text-figs. 3 and 4, one must not neglect the fact that the minimum 1:9800 (plasma) shows the same

degree of probability (Text-fig. 3), and that the minimum 1:10,200 (serum) was even more frequent than that at 1:10,500. Therefore, it seems desirable to take into account the mean values, namely, 1:9700 for plasma, and 1:10,350 for serum. These concentrations differ by about 6.3 per cent. This percentage expresses the difference in thickness of monolayers of plasma and of serum. It is assumed that the specific gravity of fibrinogen is the same as that of the other proteins of the serum, which is probably very nearly true. Hence, the thickness of the plasma monolayer is greater by 6.3 per cent in



TEXT-FIG. 4. Mean values of eleven series of measurements for serum and eleven series of measurements for plasma.

round figures than that of serum. As the "length" of the total "serum molecule," according to our former determinations,[5] is about 40.5 Ångström units, or 4.05mμ the length of the "plasma molecule" will be 43.05 Ångströms in round figures, or 4.3mμ. This difference should correspond to the amount of fibrinogen present. Two careful determinations of the fibrin content of rabbit plasma, with two different methods,[7] gave the figures 4.5 and 4.65 per cent of total proteins.

[7] Van Slyke's method, and the method consisting of weighing the dry substances of the plasma and of the serum, after dialyzing the salts out, then the fibrin, proper care being taken to remove all the salts by washing the fibrin carefully.

According to Lambling,[8] only 60 to 70 per cent of the fibrinogen is transformed into fibrin after coagulation. If 65 per cent is taken as a mean value, 4.6 per cent of fibrin corresponds to about 7 per cent of fibrinogen. The shift in the minimum, therefore, seems to be in good accord with the fibrinogen content, rather than with the fibrin content. However, it is not the intention of the writer to insist upon his figure as being a check of Lambling's statement, as too little is known at present about this subject. The error introduced through the fact that the amount of fibrinogen is expressed in per cent of the proteins and that the increase in thickness of the monolayer is expressed in per cent of total serum does not amount to more than about 0.3 per cent and is negligible in consideration of the other causes of error.

CONCLUSIONS AND SUMMARY.

Experiments are reported which indicate that a shift toward higher concentrations is observed in the minimum value of the static surface tension when plasma instead of serum solutions is used. The amount of the shift, expressed as a function of the concentration, shows that the figures are in satisfactory agreement with the determined amount of fibrinogen in the plasma.

Some evidence is given that "plasma molecules" capable of organizing themselves on adsorbing surfaces exist in plasma, and that their length would be approximately 4.3 mμ in round figures, instead of 4.0 mμ for the serum. The area occupied in the plane of adsorption by one individual molecule is, however, smaller than that occupied by the "serum molecule," thus indicating a marked structural difference between the two, the "plasma molecule" being narrower but longer than the "serum molecule." This difference may be due either to a different orientation accompanied by an increase in one of the dimensions, or else to an actual difference in structure with respect to the main axis, resulting in a decrease in the mean diameter of the "serum molecule" with an increase in the length of its main axis. The mass of the "plasma molecule" is about 6.3 per cent larger than that of the "serum molecule," in the case of rabbit serum.

[8] Lambling, E., Précis de biochimie, Paris, 1911, 250.

STUDIES IN EXPERIMENTAL EXTRACORPOREAL THROMBOSIS.

I. A Method for the Direct Observation of Extracorporeal Thrombus Formation.

By LEONARD G. ROWNTREE, M.D., and TAKUJI SHIONOYA.*

(From the Division of Medicine, Mayo Clinic, and the Mayo Foundation, Rochester, Minnesota.)

(Received for publication, April 6, 1927.)

Pulmonary embolism is one of the problems still a challenge to medicine and surgery. In the Mayo Clinic, the necropsy records show that it has been responsible for 7.3 per cent of the postoperative deaths during the last 10 years. Its annual toll throughout the world must be very large, and is probably much greater than is generally accepted, or than most statistics indicate, since pulmonary embolism is readily overlooked at necropsy unless sought for as a routine and specifically. Because of its important bearing on surgical mortality (2–11) we are making a study of thrombosis with the hope that eventually some means may be found whereby postoperative pulmonary embolism may be avoided or prevented.

In connection with this problem, one of us recalls some earlier studies carried on with John J. Abel, in which a dialyzing apparatus or an artificial kidney was used for the removal of diffusible substances from the circulating blood of living animals. In those experiments it was necessary to combat thrombosis constantly despite the fact that hirudin was employed in generous quantities as an anticoagulant. From this experience, it was thought that a similar, but simpler, apparatus might be employed advantageously in the study of the various stages of formation of thrombi, particularly that of white thrombi.

* Fellow of the Rockefeller Foundation.

Principle of the Method.

The method is simple: it consists in the introduction into the circulation of an extracorporeal vascular loop. By means of a cannula, the artery of the animal is attached to a collodion dialyzing tube, immersed in physiologic solution of sodium chloride, or other form of solution, and the blood is returned to the animal's body by another cannula attached to the vein. Before the instrument is attached to the vessels, the tubes and cannulas are completely filled with a solution of sodium chloride which approximates in its salt content the plasma of the animal, and this fluid is displaced into the body when the circulation through the apparatus is established.

During the experiment, the blood flows in a perfectly enclosed system and is returned to the body within a few seconds without having been exposed to contact with the air. On the way, it passes through the collodion tube which permits of an interchange of diffusible substances between the blood and the fluids in the outside container.

Since the inner surface of the apparatus is a foreign surface, coagulation occurs rapidly in the normal animal, within 4 to 10 minutes, but coagulation of the blood may be delayed or prevented by paraffining the tubes or by the previous injection of anticoagulants, such as heparin and hirudin, into the vein. Despite the presence of these anticoagulants, white thrombi are formed and thrombosis eventually occurs. The rate of thrombus formation can be influenced by various substances introduced into the circulation directly, or indirectly, and especially locally by dialysis through the collodion membrane, and the factors affecting each stage of clot formation may thereby be analyzed and assayed. The apparatus lends itself readily to the study of the influence of the mechanical, physical and chemical factors affecting the process of thrombosis. The experiments are carried out under complete trichloro-tertiary-butylalcohol anesthesia and are usually interrupted within 3 or 4 hours. The proportion of blood in the extracorporeal loop (about 2 cc.) is practically negligible so far as the physiology of the circulation of the animal is concerned.

The Apparatus.

The apparatus (Text-fig. 1) is simply an artificial circulatory loop permitting of extracorporeal blood flow. It consists of two parts connected by a collodion tube, which is immersed in a tube used as a container for dialyzing solutions. A glass frame holds the apparatus in place. The centrifugal and centripetal parts of the apparatus are attached respectively to the carotid artery and jugular vein. For purposes of handling and cleansing, each part is made in three sections, which are connected by means of rubber tubing. An ordinary arterial glass cannula, *a*, (about 4.5 cm. in length) is attached to the vessel and connected with the side tube *b*, which is about 6 cm. long and curved slightly to facilitate its connection with the corner tube *c* (5 cm.), which is curved in two directions, horizontally to approach the collodion tube *g*, which connects it with the other half of the apparatus, and downward to provide for immersion of the collodion membrane in the solution of the outside container *h*. The side tube and corner tube are paraffined. The collodion tube is attached to the two corner tubes by ligatures. The containing jacket is a short, stoppered test-tube with an opening in the side, which permits the immersion of the collodion tube in the solution of the dialyzing system. When the experiment is in progress, the blood flows from the artery through the cannula, side tube and corner tube of the arterial part into the collodion tube, and then into the corner tube *d*, side tube *c*, and cannula *f*, of the centripetal part back to the veins of the animal.

TEXT-FIG. 1. Extracorporeal loop.

Technic of the Experiment.

Rabbits, dogs or cats are suitable for such experiments, but throughout all our experiments rabbits have been employed; compared with dogs, they are more economical in the use of the anticoagulants.

Anesthesia is induced by the intraperitoneal injection of a saturated solution of trichloro-tertiary-butylalcohol in olive oil (from 1.5 to 2 cc. for each kilo of body weight). The apparatus is prepared, the glass side tube and corner tube paraffined, the collodion tube tied in place and the whole apparatus filled with physiologic sodium chloride solution.

The cannulas are introduced in the usual way, particular care being taken to produce as little trauma as possible. The vessels are clamped gently and carefully, to preserve, so far as possible, the integrity of the intima. With the cannulas tied in place, the cut wounds of the vessels are outside of the circuit, and hence, little, if any tissue juice or thromboplastic substance finds its way into the circulation in the extracorporeal loop. All the experiments are conducted under practically identical conditions. If anticoagulants are administered, unusual care must be exercised in the isolation and ligation of all vessels. This is necessary in the absence of clotting resulting from the anticoagulants, otherwise large quantities of blood may be lost, even from the smallest vessels.

The carotid artery and jugular vein are clamped until the apparatus is attached and then the artificial circulation is established, the blood flowing from the carotid artery into the tubes and back into the animal through the jugular vein. The blood flow can be seen clearly, particularly at points of constriction and in the neighborhood of curves. Currents, eddies and swirling motion may be obvious, particularly in the cannula where the arterial blood enters, and in the collodion tube. When doubt exists, proof of continued circulation can be readily obtained by clamping off the jugular vein and observing the presence or absence of ballooning of the vessel proximal to the clamp.

Thrombus formation can be directly observed through the collodion wall of the sac. This is more likely to occur in the neighborhood of wrinkles and irregularities in the surface of the collodion tube. Thrombi are visible first as pin-point areas, growing and radiating gradually in a direction counter to the blood stream and may attain considerable size (0.5 by 0.05 cm.). Clotting of the blood or the actual formation of the red thrombus may or may not occur, depending on the conditions of the experiment.

At will, any stage of the thrombus formation may be studied through

direct observation with the tube *in situ* or after the removal of the collodion tube. In the early stages of clotting, after the removal of the tube the surface may be gently washed with sodium chloride solution and observed directly, or studied under the microscope with or without the aid of special stains; or the specimen may be prepared and embedded in paraffin for section staining and ordinary pathologic study. The clots themselves may also be subjected to study.

DISCUSSION.

This method lends itself to the study of thrombus formation and the part played by various factors under many varying conditions. Thus the influence of mechanical, physical, physiologic, pharmacologic and pathologic factors may be determined, measured and to a certain extent assayed. Mechanically, several factors influence the rate of coagulation. The absolute and relative size of the tubes employed, the nature of the surfaces exposed, constriction, dilatation or angulation of the channel or changes in hydrostatic pressure resulting from differences in the levels of the blood stream, all influence the rate of blood flow and of coagulation. Physically, the temperature of the fluid surrounding the collodion tube is of importance. Physiologically, the vigor of the circulation and the size of the animal play some rôle. Pharmacologically,[1] drugs may be introduced which affect vigor and rate of circulation, or which directly accelerate or inhibit the process of coagulation. These may be injected intravenously, or diffusible drugs may be introduced into the extracorporeal loop locally by means of dialysis. Pathologic processes may be induced experimentally in animals, such as obstructive jaundice, chronic arsenic or phosphorus poisoning, and the influence of these diseases on the process of thrombosis determined. Finally, many combinations of these various factors may be studied simultaneously such, for instance, as the influence of transfusion or of various thromboplastic substances on the

[1] Since the phenomena of agglutination of platelets and of coagulation of blood are so imperfectly understood it has seemed better to undertake a wide range of experiments even including such as might *a priori* be expected to be fruitless. Results at variance with present hypotheses might help clarify these vexing problems.

process of coagulation in the presence of induced obstructive jaundice, or the influence of calcium on coagulation in a jaundiced or in a heparinized animal. The influence of these various factors is already under investigation and will form the basis of subsequent reports.

SUMMARY.

A new method has been described for the study *in vivo* of thrombus formation in blood circulating extracorporeally in an artificial loop. This method permits of control of many factors, and hence of intensive study of thrombus formation under many and varied conditions. It admits of separate study of the formation of white thrombi and of the deposition of fibrin, which may apparently be independent processes.

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STUDIES IN EXPERIMENTAL EXTRACORPOREAL THROMBOSIS.

II. Thrombus Formation in Normal Blood in the Extracorporeal Vascular Loop.

By TAKUJI SHIONOYA.*

(*From the Division of Medicine, Mayo Clinic, and the Mayo Foundation, Rochester, Minnesota.*)

Plate 1.

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A study of thrombosis necessitates a consideration of the theory of the clotting of blood. In this country, Howell's theory of coagulation is accepted almost exclusively. It is not my desire to enter into controversial matters either in regard to the mechanism of clotting or to the origin and nature of platelets. Howell's theory may be readily recalled from the following schema.

Coagulation Factors in the Circulating Blood:
Prothrombin, from platelets.
Antiprothrombin (heparin), from liver.
Calcium.
Fibrinogen.
The Mechanism of Clotting.—Cellular elements yield thromboplastic substances. Thromboplastic substance neutralizes antiprothrombin.
Prothrombin + calcium = thrombin.
Thrombin + fibrinogen = fibrin = clot.

From the following investigations it will be evident that the deposition of platelets and the liberation of fibrin may occur as two distinct though probably related phenomena. By the method employed it is possible to study these factors separately.

The methods employed by Zahn (6), Pitres (3), Bizzozero (1), Eberth and Schimmelbusch (2, 4) for the study of experimental

* Fellow of the Rockefeller Foundation.

thrombosis had involved observations on the mesenteric vessels of living animals after the vessels have been injured chemically or mechanically. Welch (5) used dogs chiefly, and produced thrombi of the femoral vessels and jugular vein by various procedures, such as the application of caustics, the introduction of foreign bodies and mechanical injuries. While all these methods offer excellent opportunities for the examination of injuries of very small parts of the vessels, repeated serial sections, especially during the short period of the first stage of thrombosis, are precluded, unless more than one vessel is brought into the study. With the method of investigating extracorporeal thrombosis, described in Paper I of this series, the mode and mechanics of thrombus formation, the structure of thrombi and the effects of mechanical, chemical and physical and pathologic factors on thrombus formation can be readily investigated.

Before discussing in detail the various investigations on factors influencing experimental extracorporeal thrombosis, the formation of thrombi in normal blood in the extracorporeal circulation must be discussed briefly.

EXPERIMENTAL STUDIES.

Experiments were performed according to the description in Paper I of this series, and throughout this work the same individual apparatus has been used constantly. As soon as the clamps on the vessels are released, a violent stream of blood enters the apparatus through the arterial cannula, and returns to the jugular vein by means of the venous cannula, after having passed through a collodion dialyzing tube which is immersed in a physiologic solution of sodium chloride at body temperature. Pulsations of the blood stream, eddies, currents and whirls can be easily observed through the glass and collodion walls. This is most striking in the wider part of the arterial cannula, as the stream reaches this point only after passing through the constricted part of the cannula. In the collodion tube also, streaming blood and pulsating movement can be seen before clotting occurs. Even after the latter has taken place, the pulsation of the collodion tube can be seen and felt at times. In 2 or 3 minutes a few white thrombi appear in the constricted part of the venous cannula and in the collodion tube and the blood clots quickly. This occurs also on the foreign surface

of the collodion tube and on the irregular surfaces of connecting parts of the tubing. In the collodion tube, which presents a greater caliber than other parts of the apparatus, the blood current is rather slower, the clotting begins as soon as small collections of platelets appear, and in some instances even begins before white thrombi form. At first, clotting is incomplete and leaves sufficient room for the stream to continue. White thrombi formed in the arterial part of the apparatus are dislodged by the violent current. These may lodge in the collodion tube, or be carried to the venous half of the apparatus and tend to gather especially in the constricted part of the venous cannula. The latter is an important factor in determining the duration of flow. Fibrin is formed around the platelet thrombi and soon the constricted part of the venous cannula is obstructed usually in from 4 to 10 minutes after the circulation is established (Fig. 1). Then the blood which is contained at rest in the venous half of the apparatus clots. The blood current thus becomes gradually slower and the white thrombi stick to the inner surfaces of the arterial cannula also. When complete obstruction occurs, the blood in the apparatus gradually darkens and no more ballooning of the jugular vein can be observed on clamping it below the cannula.

During the period of actual circulation, or after the obstruction is complete, the red thrombus begins to form in the jugular vein following the deposition of white thrombi in the constricted part of the venous cannula. These may grow rapidly, extending in the direction of the circulating blood. Within 30 minutes the red thrombus in the jugular vein may be 0.5 cm. long and as large as the caliber of the jugular vein. The intima of the vein may have been more or less injured by clamping or by insertion of the venous cannula, so that the thrombus may be definitely attached to the wall of the jugular vein. As the apparatus consists of sections, connecting parts leave irregular surfaces and white thrombi are formed; then fibrin forms around the thrombi; thus the mixed thrombus grows into the lumen.

More remarkable is the case in the collodion tube in the transition between the collodion tube and paraffined glass tubes. The white thrombi are laid down in ring form, at the junction of the glass and collodion tubes, where the lumen undergoes marked change. Fibrin forms in 2 or 3 minutes, and later, red clots attach to it. In other

wider parts of the tube, as soon as a few tiny white thrombi are produced, films of blood clot stick to the membrane successively. Thus a few minutes after obstruction, the collodion tube is almost filled with red clot.

In the serial sections stratification is seen in the red clot, that is, the clot is a mural red thrombus, quite flat, with a wide base developed from the tiny white thrombus. When the obstruction occurs very quickly, and the clotting time is very short, or when the blood stream is very slow, no white thrombi can be recognized. When the blood stream is very slow, the constricted part of the arterial cannula may be obstructed by white thrombi and the blood of the whole apparatus comes to standstill and clots as a column. White thrombi occasionally form in the carotid artery, starting from the injured intima or from the white thrombi in the constricted part of the arterial cannula.

Two of the seven normal rabbits weighed 2500 gm. each and presented large arteries and an unusually vigorous stream, so that circulation in the apparatus persisted for 30 minutes. In these two instances many rather large white thrombi appeared in almost all parts of the collodion tube and the venous part of the apparatus. While the vigor of the stream tends to prevent platelets from adhering to the abnormal surfaces of the apparatus and to prevent fibrin from being deposited on the surfaces of white thrombi, the stream in the venous cannula, on the other hand, is not powerful enough to wash away the white thrombi gathered there. The collodion tube in these animals presented a thin layer of clot at its bottom, and ring forms of large mixed thrombi at the points of connection. But true obstruction takes place always in the constricted parts of the cannulas sooner or later, owing to the mixed thrombi formed either in the venous or in the arterial cannula, the exact site depending largely on the vigor of the blood stream.

SUMMARY.

Thrombus formation has been studied in normal rabbits with an experimental method of establishing extracorporeal circulation. In the normal animal circulation in the extracorporeal loop usually ceases in from 6 to 10 minutes, or at most, in very large and vigorous

animals, in 25 minutes.[1] Cessation of the circulation is due most frequently to obstruction of the venous cannula (sometimes of the arterial cannula) by a mass of white thrombi and secondary fibrin formation around it. The site of the clot is determined somewhat by the swiftness of the blood stream.

In the collodion tube red mural thrombi are obtainable as a rule. They are flat and present a wide base resting on tiny white thrombi. After the complete obstruction of the circulation, the blood in the apparatus clots very rapidly. When the obstruction occurs very quickly either in the arterial or venous cannula, or when the blood stream is very slow, the clotting in the loop may occur before platelets are laid down in large numbers or before the formation of the white thrombi is evident.

Mixed thrombi are found in the jugular vein, and have their inception in the white thrombi, in the cannula or from the injured intima. They extend in the direction of the blood flow.

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EXPLANATION OF PLATE 1.

FIG. 1. White thrombus mass with red clot in the venous cannula (in the case of normal blood at the end of 10 minutes). *a*, mass of white thrombi. *b*, red clot. × 100.

[1] This is true when only the side and corner tubes are paraffined. When all glass tubes are paraffined, circulation is maintained for a number of hours, as will appear in a future communication.

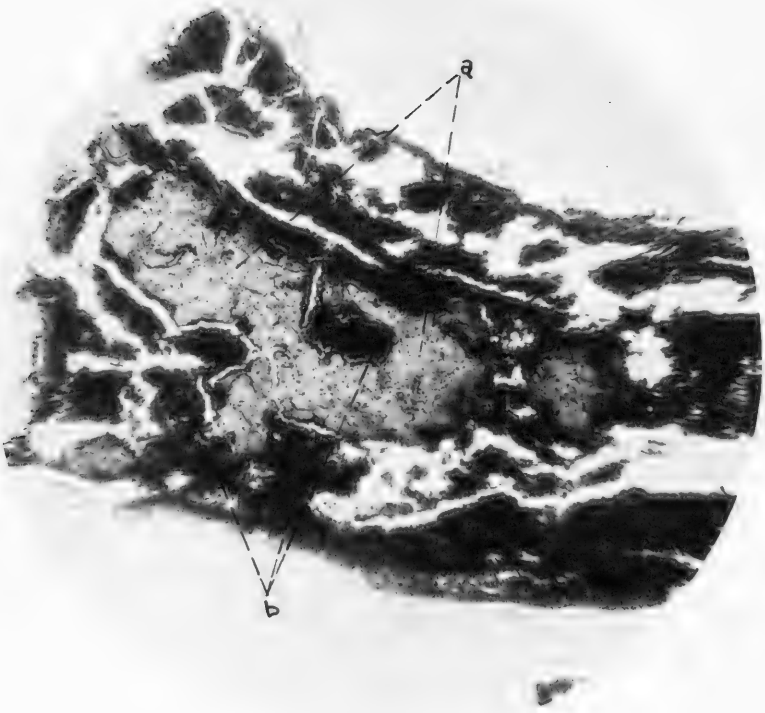


FIG. 1.

STUDIES IN EXPERIMENTAL EXTRACORPOREAL THROMBOSIS.

III. Effects of Certain Anticoagulants (Heparin and Hirudin) on Extracorporeal Thrombosis and on the Mechanism of Thrombus Formation.

By TAKUJI SHIONOYA.*

(From the Division of Medicine, Mayo Clinic, and the Mayo Foundation, Rochester, Minnesota.)

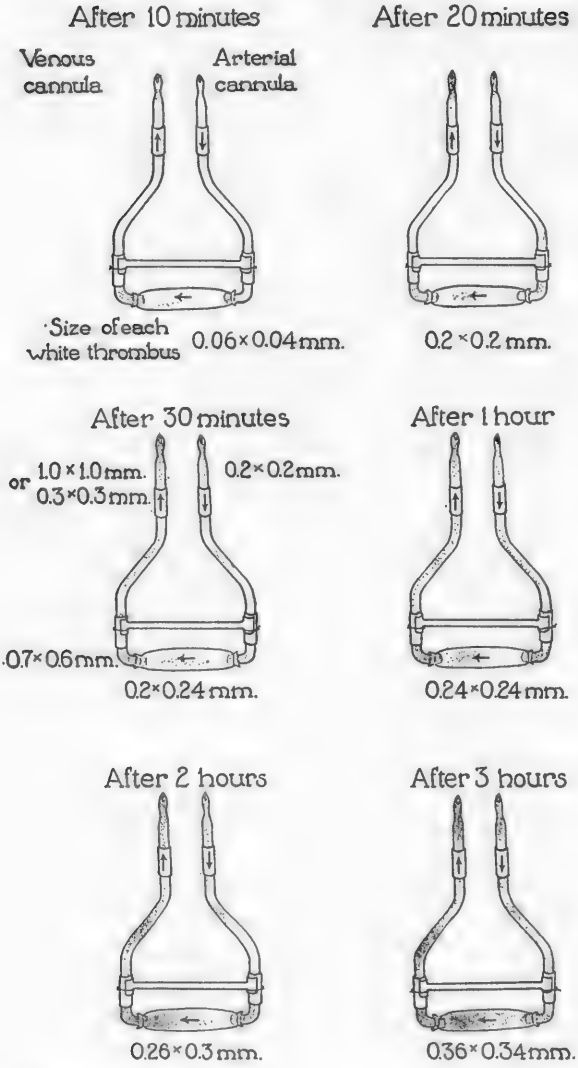
Plates 2 and 3.

(Received for publication, April 6, 1927.)

Welch (9) describes the steps in the formation of a thrombus after injury to the vessel wall, as follows: There is an accumulation of blood platelets adhering to the wall at the point of injury; leucocytes, which may at first be present in small numbers, increase rapidly, collecting at the margins of the platelet masses and between them. Not until the leucocytes have accumulated in considerable numbers, does the fibrin appear. Therefore, the formation of fibrin is secondary. Nevertheless, the fibrin formation plays a great part in the growth of red thrombi and the occurrence of obstruction and of intravascular clotting.

The effects of anticoagulants on the process of thrombosis with the new method of investigation of extracorporeal thrombosis was studied. Interest centers especially on the study of the mechanics of thrombosis and the preventive or retarding influence of anticoagulants which prohibit clotting for long periods. Here heparin, an antiprothrombin introduced by Howell (3), has been employed mainly. Howell (4) states that heparin is the anticoagulant always present in normal blood, and that it may be used intravascularly without injurious effects. Reed (7) found that repeated injections of this material always result in an increase of clotting time. In my experiments the heparin used (Hynson, Westcott and Dunning) was

* Fellow of the Rockefeller Foundation.



TEXT-FIG. 1. Progression in formation of white thrombi.

standardized, 1 mg.[1] rendering 5 cc. of cat blood incoagulable *in vitro* for 24 hours. It is dissolved in physiologic sodium chloride solution, 20 mg. to 1 cc., and is injected intravenously in doses of 20 to 25 mg. for each kilo of body weight.

Experimental Studies with Heparin.

Rabbits weighing about 2 kilos were anesthetized, and the carotid artery and jugular vein of the same or opposite sides cautiously exposed for more than 1 cm. All small vessels needing to be cut were first ligated to prevent subsequent loss of the blood rendered incoagulable by heparin injection. Then the anticoagulant was injected and the apparatus for extracorporeal thrombosis connected with the vessels of the animal. Since physiologic sodium chloride solution is usually regarded as an indifferent medium so far as blood is concerned, it was used in the container in which the collodion tube was immersed, as a control or standard experiment. The clotting time of the blood from the ear was tested often during the experiment by Mills' method; immediately after injection and persisting for about 4 hours the clotting time may be 30 minutes, or perhaps several hours. 4 and sometimes 5 hours after the injection of this amount of heparin the clotting time of the blood became shorter than 30 minutes, and tended to decrease rather quickly: for instance, in the next half hour, it became normal or sometimes less than normal.

There was considerable uniformity in the formation of white thrombi. From the results of thirty experiments, the sequence of events (Text-fig. 1) was as follows: After 5 minutes, no white thrombi were visible macroscopically, but a few small white thrombi were seen microscopically, which were chiefly composed of clumps of platelets, situated along the wrinkles of the bottom of the collodion tube. In size these clumps measured 0.06 by 0.04 or 0.04 by 0.04 mm. On and around the platelet clumps a few leucocytes were recognized. After 10 minutes numerous tiny white thrombi were seen macroscopically in the venous corner tube, or on one of the curved surfaces. In the collodion tube the number of white thrombi appeared increased, but they were still recognized only under the microscope. Also a few tiny ones were found in the venous cannula. After 15 or 20 minutes a few tiny white thrombi appeared in the arterial corner tube, while those in the venous cannula and corner

[1] 1 mg. of pure heparin renders 100 cc. of human blood incoagulable for 24 hours or more (Howell's personal communication).

tube increased in size and number. After 25 or 30 minutes numerous small white thrombi became macroscopically visible in the arterial cannula and in the curves of the side tubes, but were fewer on the arterial side. Those in the other parts increased in number and size. Sometimes the whole inner surface of the venous corner tube was coated with a number of white thrombi which were connected by threads of fibrin. The thrombi in the venous cannula and corner tube may be large (0.7 or 0.6 mm. in diameter); those in the cannula almost obstructed the constricted part. The blood stream in the apparatus gradually became slower, the laying down of the platelets was relatively decreased, and so the further growth of the white thrombi was relatively decreased. After 2 hours the thrombi had increased in size and number and the narrow part of the venous cannula was almost obstructed. In the collodion tube, many large white thrombi were obtainable (Fig. 1), often in the form of fern-like radiations, in the venous half or arterial half or sometimes in the center of the bottom of the collodion tube. These were propagated against the current. The site and growth of the white thrombi in the apparatus are shown in Text-fig. 1. The following protocol is typical of what was generally observed:

At 11.10 a.m. Rabbit 10, weighing 1400 gm., was injected intravenously with 40 mg. of heparin. At 12.00 the apparatus was connected and the collodion tube surrounded with physiologic sodium chloride solution of body temperature. The clotting time *in vitro* was more than 30 minutes. At 2.00 the apparatus was disconnected and the collodion tube detached. The blood remained totally incoagulable. All the surfaces of the apparatus were gently washed with physiologic sodium chloride solution. In the tubes of the venous side, numerous white thrombi were seen with a smaller number in the arterial tubes. The venous cannula seemed to be almost obstructed by a mass of white thrombi. In the collodion tube many white thrombi were observed in a fern-like radiating mass, which seen microscopically in side view appeared like a succession of hills and valleys. The size of the individual thrombus varies. Thus of thirteen thrombi seen under low power in one microscopic field, one thrombus measured 0.20 by 0.20 mm., four measured 0.16 by 0.12 mm., and eight 0.06 by 0.04 mm.

Experiments with Hirudin.

When hirudin was used instead of heparin, in a dosage of 10 mg. for each kilo of body weight in three experiments, the results were almost the same as when heparin was used (Fig. 2).

The Mechanism of Thrombosis and the Influence of Heparin.

These findings in the tubes should be discussed in connection with the mechanics of the blood flow. The blood in the carotid artery carries many platelets and leucocytes, which are hurled against the narrow part of the glass cannula. Platelets and leucocytes may adhere to the wall, but as the current is rapid, they usually become dislodged. The narrow part of the arterial corner tube is, as a rule, less often obstructed than that of the venous cannula, and then only at a later period. Wherever whirlpools, eddies and stagnation occur, platelets come in contact with foreign surfaces and they are agglutinated and white thrombi tend to form. At the spots where eddies form, relatively stagnant areas of blood are found; these favor agglutination probably through surface changes in the platelets.

The investigations show that in every part of the apparatus, even on the paraffined surface, the early stages of thrombosis can be demonstrated; though they appear earlier and are more marked, on the rough, irregular surface, for example, on the wrinkles of the collodion membrane. In the widest part of the apparatus, eddies occur on the surface in contact with the collodion and the platelets settle down.

The fact that clumps often assume a radiate or fern-like form in the lower part of the venous half of the collodion tube where whirlpools are seen through the collodion membrane, supports the von Recklinghausen theory (6). Eberth and Schimmelbusch's view (2, 8) in this regard commands attention but does not sufficiently explain the formation of platelet thrombi. Von Recklinghausen's theory appears more acceptable in explaining the mechanics of thrombosis. But in these experiments in the ampulla-like widening of the vessel, the thrombus forms mainly in the distal part of it and rarely in the proximal part. Platelets accumulate more in the distal part of the collodion tube than in the proximal end of it. Therefore, the explanation of the formation of the platelet clumps is to be found in the movement of the blood stream, rich in platelets and adequately slow, washing against the pathologic inner wall of the vessels, stagnating there and breaking into eddying currents against the irregular rough surface. If the stream is much slower, the white thrombi are chiefly formed in the arterial part of the apparatus; for instance in the collodion

tube it takes place in the arterial half of the lower part near the mouth of the corner tube. This is exactly in accord with the results of the experiments of Aschoff (1) and his coworkers with sand, showing that if the sand stream from the smaller vessel flows very slowly, a sand bank is formed in the bottom of its mouth.

The Use of Anticoagulant as an Antithrombotic Substance.

As Mason (5) has already stated, heparin might be effective against certain kinds of thrombosis, considering the thrombosis as a kind of intravascular clotting of the blood. But, since the first stage of autochthonous thrombosis is the deposition of platelets and not the deposition of fibrin, it does not suffice. At least single doses of the heparin used (Hynson, Westcott and Dunning) did not prevent the formation of white thrombi on foreign surfaces. In spite of the administration of a sufficient dose of this anticoagulant, and in spite of the incoagulability of the blood *in vitro*, white thrombi appear at the site where the conditions were conducive to clotting. The white thrombi gradually grew and sooner or later obstructed the lumen of the apparatus, and still later the lumen of the vessel. Therefore it appears that anticoagulants such as heparin[2] and hirudin in their form and in single doses do not afford complete protection against thrombosis. Experiments involving the continuous administration of heparin are contemplated.

In order to examine the structure of thrombi formed during the period of effective action of the anticoagulant, the apparatus itself may be removed and the ligatures holding the collodion tube in place may be cut at any desired time during the experiment. Thus the collodion tube with its contents is removed, its upper wall is carefully slit with a pair of delicate scissors and its inner surface cautiously washed with physiologic sodium chloride solution. Like a glass slide the collodion membrane is translucent enough to be examined directly under the microscope.

It is not desirable, perhaps, to dwell further on the character of the structure of the thrombus because that would but repeat Welch's

[2] The heparin used was supplied by Hynson, Westcott and Dunning. Professor Howell believes that pure heparin may entirely prevent the formation of white thrombi.

description (Figs. 3 and 4). Suffice it to say that his findings as to the structure of the white thrombi were verified by the method used in these experiments as well in serial sections as in the fresh state. But there is one respect in which my results appear to differ from his: the formation of fibrin is postponed on account of the anticoagulant action.

CONCLUSIONS.

1. Effects of anticoagulants, heparin and hirudin, on extracorporeal thrombosis were studied in thirty-three experiments by means of the extracorporeal vascular loop. In spite of adequate single doses of the anticoagulants white thrombi are formed and obstruction to flow may follow in the course of time, but the formation of red thrombi is markedly retarded.

2. The new method throws some light on the mechanics of thrombosis. The influences of foreign surfaces, of irregularities on the surface of the vessels, of whirlpools and of eddying motion of the blood current and of slowing and of stagnation with consequent prolonged contact with foreign surfaces are demonstrated, and these are analyzed in respect to the laying down of platelets and leucocytes.

3. The structure of white thrombi formed after anticoagulant injection is almost identical with that described by Welch. In one respect, however, the present results appear to differ from his: the formation of fibrin is postponed and retarded, and hence the collections of platelets are deposited in greater amount.

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EXPLANATION OF PLATES.

PLATE 2.

FIG. 1. Growths of white thrombi after heparin injection. *a*, after 10 minutes white thrombi appeared as a white line on the wrinkle of the center of the collodion tube. *b*, after 30 minutes the white thrombi grow up more remarkably. *c*, after 2 hours white thrombi multiply and are laid down in radiating form. *d*, after 3 hours.

FIG. 2. White thrombi after hirudin injection. *a*, after 2 hours. *b*, after 1 hour.

PLATE 3.

FIG. 3. Hillocks of platelet thrombi 1 hour after anticoagulant injection. *a*, clumps of platelets. *b*, fibrin with white cells.

FIG. 4. Hillocks of platelet thrombi 3 hours after anticoagulant injection. *a*, clumps of platelets. *b*, fibrin with white cells.

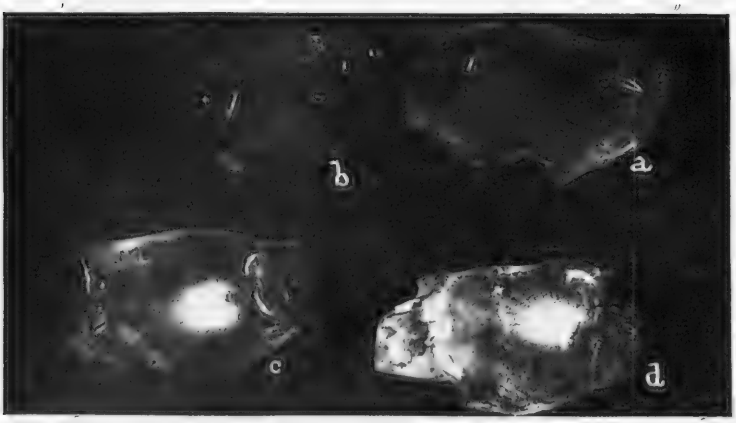

FIG. 1.

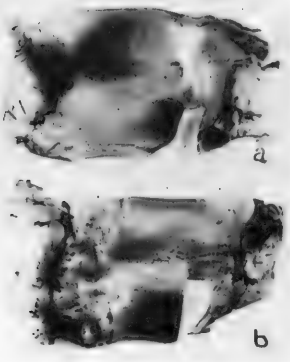

FIG. 2.

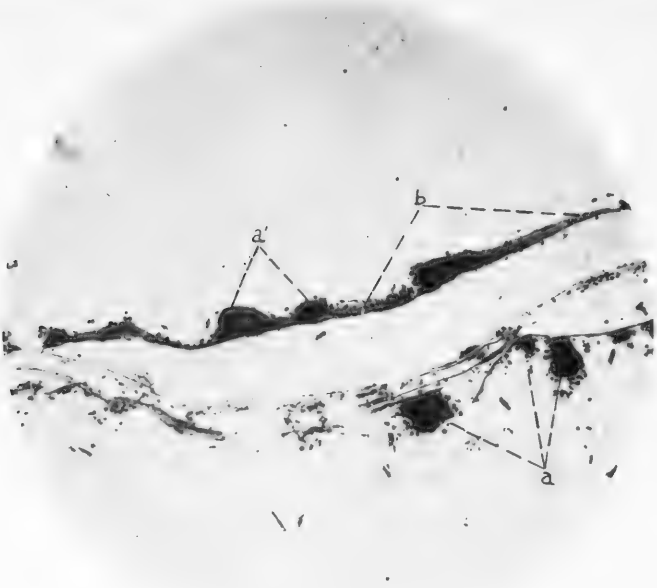

FIG. 3.

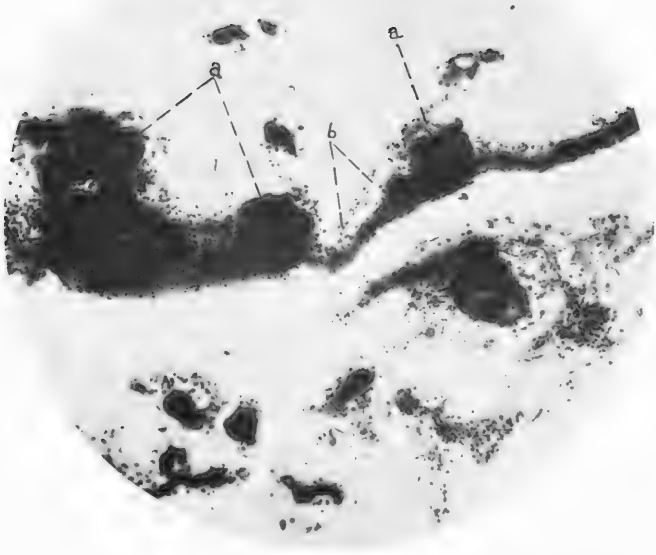

FIG. 4

DIET AND TISSUE GROWTH.

V. The Effect of Dietary Protein on the Remaining Kidney of Adult White Rats Following a Unilateral Nephrectomy.

By THEODORE S. MOISE, M.D., and ARTHUR H. SMITH, Ph.D.

(*From the Department of Surgery and the Laboratory of Physiological Chemistry, Yale University, New Haven.*)

Plates 4 and 5.

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INTRODUCTION.

The recent literature contains an increasing number of accounts of investigations on the effect of the ingestion of excessive amounts of protein on the kidneys of experimental animals. There is general agreement on the observation of a physiologic enlargement but conflicting results are reported concerning the part played by protein in the production of pathologic renal lesions.

The character of the physiologic enlargement has been the subject of prolonged dispute. The literature on this question has been summarized by Arataki (1926, *a*) who reported from his own studies on rats that there is no increase in the number of glomerular unit systems but that the enlargement consists in an increase in the size of both the glomeruli and tubules, an hypertrophy, together with an actual cellular increase of the interstitial tissue, an hyperplasia. He believes in addition that there is an hyperplasia of the constituent cells of the glomeruli and tubules. Furthermore he observed that the enlargement is incomplete after 80 days, and stated that in some instances it may approximate 100 per cent.

Although there is no increase in the number of glomeruli in the rat, in response to feeding excessive amounts of protein, the formation of these structures continues throughout the first 100 days of life according to Arataki (1926, *b*). He stated further that the total number of glomeruli at birth is about 10,000, and at the age of 25 days about 25,000, and that by the 100th day the maximum of 30,000 has been attained. This level is maintained to the age of 350 days but by the 500th day it has fallen to 22,000.

In a study of the compensatory enlargement of the opposite kidney after ligation of one ureter in white rats Hinman (1923) has stated that the histologic

changes of compensatory renal hypertrophy may be divided into three stages: first, initial congestion; second, active mitosis and growth, and third, the final equilibrium of hypertrophy. He mentioned further that the increase in the size of the intact kidney is complete after an average of 20 to 30 days; the average total increase is 20 per cent of the normal and doubling of the renal tissue is never observed. The question of diet is not discussed in either of the above papers.

Smith and Moise (1927) have reported an extensive series of experiments on the relation of the protein constituent of the diet to the rate and degree of compensatory enlargement of the remaining kidney after unilateral nephrectomy in adult white rats. These authors have observed that on the "standard" diet containing a moderate concentration of protein (18 per cent, Table I) there is a rapid increase (24 per cent) in the compensatory enlargement of the remaining kidney within the

TABLE I.

Composition of Experimental Diets.

	"Standard food"		"High protein" food	
	Part of diet	Part of total calories	Part of diet	Part of total calories
	per cent	*per cent*	*per cent*	*per cent*
Protein (casein)[1]	18	14	85	76
Carbohydrate (raw cornstarch)	51	39		
Fat { lard	22	47	7	24
Fat { cod liver oil	5		4	
Salt mixture[2]	4	4	4	
700 mg. dried yeast daily given with each of the diets				

[1] A commercial product containing 13 per cent nitrogen.

[2] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557.

first 3 weeks. A similar series on a high protein diet (85 per cent casein) showed an extremely rapid gain to 63 per cent in 21 days. The curves of enlargement on the two diets continue approximately parallel and at 150 days the average enlargements are 48 and 121 per cent for the standard and high protein diets respectively. When adult white rats were fed diets containing increasing concentrations of protein, observations at a constant time interval (21 days) after unilateral nephrectomy showed that the increase in the size of the remaining kidney is directly proportional to the protein content of the food. The values vary from 24 per cent with the standard ration to 77 per cent with the high protein diet. All of the diets used are adequate for maintenance and growth. The large proportion of calories obtained from protein in some of the rations is the variable factor.

The material obtained from this investigation (Smith and Moise, 1927) affords a unique opportunity of studying the histologic changes in a large series of kidneys not only performing approximately double functional duty owing to the previous unilateral nephrectomy but also subjected to the additional burden of eliminating the metabolites of an extremely high protein diet (Table I). The purpose of the present communication is to report such structural changes and if possible to explain the discrepancy in the reports of other investigators.

In earlier studies of a somewhat similar nature (Newburgh, 1919), egg white, casein and soy beans were fed to three different groups of rabbits for periods of time varying from a few days to 7 months. Examination of the kidneys revealed evidence of acute and chronic renal disease, consisting chiefly of casts in tubules, varying degrees of injury to tubular epithelium and scarring of the cortex. There were no marked glomerular lesions. Newburgh and Clarkson (1923) observed marked dilatation of the tubules of the kidneys of rabbits fed diets containing lean beef amounting to 27 and 37 per cent of the ration for several months. There were no marked glomerular lesions. They called attention to the difference between the focal cortical scars occurring spontaneously in rabbits and the lesions observed in their experiments.

Evans and Risley (1925) described definite tubular and glomerular lesions in white rats fed a variety of substances containing between 27 and 75 per cent protein for periods from 6 to 15 months but the diets used in their studies were admittedly inadequate in salts and vitamines.

In all of the above mentioned experiments the diets were not only extreme but also inadequate and furthermore the early studies on rabbits (Newburgh, 1919) have been criticised because of the common occurrence of spontaneous lesions in this animal. Polvogt, McCollum and Simmonds (1923) made similar studies on white rats, with diets adequate for maintenance and growth and containing between 31 and 41 per cent protein. The time of feeding varied from a minimum of 129 to a maximum of 485 days. Congestion, degeneration of the tubular epithelium, casts and cellular detritus in the lumina of the tubules, hyaline material within the capsular space and adhesions between the glomerular tuft and Bowman's capsule were described in the kidneys of these animals.

Osborne, Mendel, Park and Winternitz (1927) observed tubular and glomerular lesions in the kidneys of eighteen white rats that had been fed high protein diets for long periods of time. These authors state that the lesions were focal in nature and were exceedingly slight in the entire group with the exception of seven animals, five of which were on a 40 per cent casein diet for 400 days or more. Exactly similar lesions were observed in five of the control animals maintained on Sherman's stock diets for over 500 days. The series also included eighteen rats on high protein diets (60 to 95 per cent) for periods varying from 200 to 360 days in which no renal lesions were observed. The average age of the animals on the high protein diet showing lesions was 348 days while those showing the lesions described as "severe" were over 400 days old.

On the other hand numerous investigators (Osborne, Mendel, Park and Darrow (1923), Osborne, Mendel, Park and Winternitz in a preliminary report (1925), Drummond, Crowden and Hill (1922), Reader and Drummond (1925), Miller (1925), Jackson and Riggs (1926), Anderson (1926), Addis, MacKay and MacKay (1926) and Kennedy (1926)) have reported that no pathologic structural changes were observed in the kidneys of intact animals (white rats, rabbits and cats) maintained on high protein diets for relatively prolonged periods. There is thus confusion in the literature on a point which is not only of academic interest but also of clinical importance.

EXPERIMENTAL.

Immediately following a right nephrectomy the animal was placed on the diet to be used and after a definite time interval the remaining kidney was removed for study. All of the kidneys were accurately weighed for determination of gross enlargement. Some were used for the determination of total solids while for the present study a large number were fixed in 10 per cent formalin, sectioned and stained with hemotoxylin and eosin.

The findings are based on the study of histologic sections from over 200 adult rats; namely, the left kidney from 92 animals on the "high protein" diet (85 per cent casein) and from a slightly smaller number of animals on the standard diet (18 per cent casein), for periods varying from 3 to 150 days after the control nephrectomy. The kidneys from another series of 60 animals on six diets varying in protein content from 30 per cent to 90 per cent casein for a period of 21 days following a right nephrectomy were also sectioned for histologic study. The animals were all somewhat under a year old (approximately 275 to 350 days) at the end of the experimental period.

In addition to the series of animals on the 18 per cent casein ration, the kidneys from groups of rats maintained on Sherman's diets "A" and "B" for periods between 350 and 565 days were examined.* As further controls the removed right kidney from many of the experimental animals was available.

Although there is no significant anatomic evidence of renal injury in the animals on the standard diet, the kidneys of the rats on the "high protein" diet show some interesting structural changes. In the early and middle periods, excluding the phenomenon of gross enlargement, the observed changes are relatively inconsequential. However, in the late periods, namely, after 90, 120 and 150 days the kidneys of the animals on the high protein ration show significant glomerular and tubular changes (Table II).

* The authors are greatly indebted to Professor E. A. Park of the Department of Pediatrics, Yale University, and Professor H. C. Sherman, Columbia University, for the material included in this group.

These changes represent actual lesions of the kidney and were observed in all animals on the high protein ration for 90 days or more. The lesions were not merely isolated focal changes but were conspicu-

TABLE II.

Data on Rats Fed High Protein Diet and Showing Renal Lesions.

Rat No.	Age completion experiment	Initial body weight	Final body weight	Right kidney weight	Left kidney weight	Remarks
Interval after nephrectomy—90 days						
		gm.	*gm.*	*gm.*	*gm.*	
594[1]	292	310	320	1.225	3.205	
597	292	292	262	1.202	2.517	
602	295	279	268	1.239	2.321	
603	295	298	300	1.173	2.774	
606	295	309	300	1.250	2.865	
608	295	300	293	1.196	2.538	
Average[2]...		288	271	1.171	2.389 Enlargement 113 per cent	
Interval after nephrectomy—120 days						
562	317	280	288	1.120	2.558	
580	317	282	262	1.132	2.244	
584	317	272	241	1.108	3.504	
590	322	293	285	1.287	2.529	
592	322	293	251	1.384	2.266	
Average[2]...		288	276	1.205	2.608 Enlargement 123 per cent	
Interval after nephrectomy—150 days						
564	346	293	290	1.086	2.429	
568	346	299	284	1.121	2.381	
569	346	266	262	0.978	2.097	
575	348	291	271	1.190	2.400	
576	348	289	290	1.111	2.435	
578[1]	348	284	214	1.106	1.912	
Average[2]...		287	277	1.126	2.419 Enlargement 121 per cent	

The kidneys of the animals listed in this table (all rats maintained for 90 days or longer on the 85 per cent protein diet with the exception of those utilized for the total solid determinations) show the renal changes described above

These lesions become progressively more numerous and more widespread in the 90, 120 and 150 day periods

The second group in the 150 day interval (age 271 days) showed less enlargement and slightly less evidence of renal damage than was observed in the first group

TABLE II—*Continued.*

Rat No.	Age completion experiment	Initial body weight	Final body weight	Right kidney weight	Left kidney weight	24 hr. urine protein 120–140 days	Casts
				Interval after nephrectomy—150 days			
		gm.	*gm.*	*gm.*	*gm.*	*mg.*	
667	271	245	184	1.147	1.718	119	Present
668	271	263	285	1.184	2.185	49	"
669	271	270	282	1.406	2.276	45	"
670	271	277	320	1.130	2.359	63	"
671	271	273	280	1.145	2.471	18	Absent
672	271	282	318	1.144	2.448	45	Present
673	271	261	238	1.012	1.928	70	"
674	271	257	315	1.176	2.541	84	"
675	271	234	291	0.981	1.968	60	"
676	271	243	271	1.008	1.827	40	"
Average...		261	278	1.133	2.172 Enlargement 79 per cent		

[1] Eliminated from computations of enlargement on account of large abscess (Rat 594) and marked emaciation (Rat 578).

[2] The average values for body weight and kidney weight are based on data from rats used for computation of total solids in addition to those listed in this table. For method of calculating per cent of renal enlargement see Smith, A. H., and Moise, T. S., *J. Exp.* Med., 1927, xlv, 263.

ous and relatively widespread becoming progressively more marked in the 90, 120 and 150 day periods. The structures included in an injured area are frequently radial in form suggesting the involvement of individual glomerular unit systems.

The changes in the glomeruli consist in serum in the capsular spaces, proliferation of the epithelium of Bowman's capsule with and without adhesions between the tuft and capsule, fibrous thickening of Bowman's capsule, partial fibrosis of the glomerular tuft and in many instances infiltration of round cells in and around these areas (Figs. 1 to 4).

In the same periods, 90, 120 and 150 days, the tubules show a rather conspicuous desquamation of the lining epithelium with many fairly well preserved epithelial cells and amorphous material within the lumina.

There is a slight general tubular enlargement with a somewhat diffuse patchy distribution of areas of marked tubular dilatation. In these dilated tubules the epithelium may form a single relatively flat layer or may appear in many layers as projections into the tubules and in some instances practically fills the lumina. In may such tubules the lining epithelium consists of recently formed cells in which mitotic figures are seen as evidence of active epithelial proliferation. There are many focal groups of dilated tubules with absent or markedly flattened epithelial lining cells showing no evidence of cellular activity. In some dilated tubules hyaline casts are seen. There are other areas showing an increase in the interstitial tissue with a variable amount of round cell infiltration. There are no red blood cells in the tubules or the capsule spaces (Figs. 5 and 6).

These changes are chiefly late manifestations of the injury or irritation to which the kidney has been subjected. It seems probable that the irritation produces slight changes in the early periods but that the methods of study only demonstrate the later aspects of the process. The findings in a single animal on a 75 per cent casein diet for 21 days after a right nephrectomy suggest the correctness of this hypothesis. In the sections of the left kidney from this animal showing an enlargement of 96 per cent a few glomeruli are seen in which the tufts are large with widely separated cells. In some instances the tuft and capsule are in actual apposition or the space is bridged by strands of fibrin. These findings suggest fresh adhesions and are possible precursors of the fibrous adhesions observed in the late periods. Such apparently fresh adhesions are also seen in the sections of the kidneys 150 days after nephrectomy.

In addition to these actual lesions of the kidney there are other changes of a physiologic nature. In the normal rat's kidney a few glomeruli are seen in which the tubular epithelium extends into and partially lines the glomerular sac. This condition is observed more frequently in the high protein animals and may involve fully one-half of the circumference of Bowman's capsule. It seems probable that this is a compensatory phenomenon whereby the tubular epithelium, in response to a functional demand, partially replaces the normal lining of Bowman's capsule.

In the early periods in spite of the extraordinarily rapid renal en-

largement, there is no histologic evidence of cellular proliferation in the kidneys of the animals on the high protein régime. In the late periods the physiologic enlargement is apparent in the increase in the size of the individual constituent parts of the kidney. Although no actual measurements were made the tubules are considerably dilated in all rats subsisting on the high protein diets for 90 days or more after unilateral nephrectomy. In some animals this dilatation was very striking. The tubular epithelial cells and the glomerular tufts and sacs also appeared to be larger in the high protein animals after 90 days. In the animals on the high protein diet for shorter periods of time the enlargement of the constituent parts of the organ was less evident but can probably be demonstrated by actual measurements. There was no evidence of infection in the kidneys of any of the experimental animals.

The urinary albumin excretion was studied as a supplement to the histologic findings referred to above. It is known that intact rats on a mixed adequate diet constantly show small quantities of protein in the urine (see Nelson and McCay, 1924, and Jackson and Riggs, 1926) and the aim in the present investigation has been to make a comparative study of the albuminuria in the rats fed the high protein diet and those fed the control food. The urine was examined for casts at the same time as the protein determinations were made.

The rats used were males, 120 days old at the time of the initial nephrectomy, and the determinations were made at short intervals from 25 days to 143 days after the operation. Daily urine collections were made over a period of 4 days. The rat was removed from the metabolism cage for 1 hour in the morning and 1 hour in the afternoon for feeding in order to prevent the contamination of the urine samples by food spilled within the metabolism cage.

The rat excretes a very concentrated urine and it is possible that some of the sample was lost by its drying on the metal parts of the cage with which it came in contact. The results, while they may not represent an accurate 24 hour collection, are, however, comparable.

In most of the determinations the samples as collected were made up to 50 cc. with distilled water. To 10 cc. of the filtered diluted urine in a graduated centrifuge tube was added 3 cc. 10 per cent potassium ferrocyanide and 2 cc. 50 per cent acetic acid. After mixing and allowing to stand for 10 minutes it was centrifuged at 1600 R. P. M., for 10 minutes, the volume of precipitate was read off directly and calculated on the basis of the 24 hour excretion. The method was checked with nitrogen determinations on the heat coagulum of the urine and a curve plotted correlating the ferrocyanide precipitate with mg. of protein.

The data are summarized in Table III. In order to obtain a large enough number of observations to permit statistical treatment, the results were grouped into four periods depending on the time interval after the nephrectomy. Although this grouping is arbitrary, it is perfectly obvious that at any period chosen after 60 days, the animals on the protein-rich food excrete significantly larger quantities of albumin than the rats on the standard ration.

There is a tendency for the values for the latter group to decrease with time after nephrectomy while in the rats in the former group the

TABLE III.

Correlation of the Protein Excretion in Urine with Diet.

Period.....		I	II	III	IV
No. of rats	Standard diet	6	4	9	11
	High protein diet	7	4	10	13
No. of determinations	Standard diet	31	16	43	63
	High protein diet	30	17	67	53
Interval after nephrectomy, *days*	Standard diet	25,37,53	64,72	88,101,107 109,114	122,125, 143
	High protein diet	25,34,40, 53	61,68	88,98,101, 104,119	122,125, 140
Average value of 24 hr. urine protein, *mg.*	Standard diet	33	35	28	18
	High protein diet	31	56	63	63

Average value of 24 hour urine protein in six intact rats on standard ration was 32 mg.

albumin values increase to a level reached at about 90 days. As an additional control, six intact rats were fed the standard food and the albumin excretion measured. The value for the 24 hour elimination, namely 32 mg., is practically the same as those of the above mentioned animals in Period I.

Jackson and Riggs (1926) found a variation of 4 to 21 mg. daily in normal rats. Data for comparison are also available on eight animals nephrectomized at 200 days of age. In a time interval after the operation corresponding to Period II in the table, the average of

thirty-four determinations was 49 mg., a value which corresponds fairly well with the data on the younger rats.

Samples of urine for casts were obtained directly from the urethral orifice on a slide by causing the rat to breathe ether vapor.

In Periods III and IV, those intervals when structural damage was observed, 112 samples from 13 rats on the protein-rich diet, casts were observed in 33 cases on 12 animals while in 107 samples from 11 rats on the control food, only 3 instances of casts were seen in 2 animals. Three observations of casts were made in the former group of rats in Periods I and II while none were found in the latter group. The increased excretion of protein in the urine and the greater incidence of casts furnish striking corroborative evidence for renal injury in the remaining kidney of the animals on the high protein diet.

DISCUSSION.

The necessity for adequate control in the differentiation of spontaneous renal disease and changes attributed to any experimental procedure is quite obvious and accordingly it is advisable to mention the occurrence of spontaneous renal lesions in rats. In a study of wild rats, Ophüls and McCoy (1912) have noted the common occurrence of a characteristic type of nephritis. Reader and Drummond (1925) observe that focal renal lesions are not uncommon in white rats. In the present investigation the occurrence of such changes in normal animals has been recognized.

There is little doubt that the structural changes observed in the animals on the high protein diet are a result of the experimental procedure and are not purely a part of the spontaneous renal changes that occur in white rats. In the present experiments actual renal lesions, including a proliferation of the tubular and capsular epithelium, adhesions between and a partial fibrosis of the glomerular tuft and capsule, were observed in animals maintained for 90 days or more after a unilateral nephrectomy on a diet containing 85 per cent casein. The kidneys of the animals on the high protein ration also showed marked tubular dilatation, including a rather diffuse change with evidence of active regeneration of the epithelium as well as other areas apparently atrophic in nature, showing groups of dilated tubules with a flattening or absence of the epithelium and without evidence of regeneration.

These latter groups of dilated tubules may be secondary to an injured glomerulus.

The lesions observed in animals fed the high protein food are widespread in distribution in contrast to the relatively inconspicuous focal lesions observed in the control animals. Occasional scattered focal lesions are not infrequently observed in normal adult white rats more than a year old and accordingly in the interpretation of our data no significance has been attached to isolated focal areas, of tubular dilatation, and, less frequently, of glomerular adhesions and capsular thickening. They are apparently phenomena of senescence and were observed regardless of the composition of the diet and became progressively more common with age. In the kidneys of control animals maintained on a normal dietary régime for 120 and 271 days respectively no focal lesions were found. Observations were made on the kidneys of rats maintained on Sherman's diets "A" and "B" for 350, 361, 372, 414 and between 500 and 565 days. At 350 days these focal lesions were inconspicuous or entirely absent but became progressively more common with successive periods. After 500 days they were present in a high percentage of animals. It is interesting to note in this connection that Arataki has observed a normal decrease in the total number of glomeruli with advancing age and it is possible that the spontaneous focal lesions are secondary to the normal process of glomerular involution.

In the experimental animals maintained on the standard ration such changes were practically never seen while in the animals on the high protein régime the focal lesions were larger and more frequently observed than in normal rats.

In searching for an explanation of the disagreement between the results reported by other investigators the important factors, namely, the animal, the diet and the duration of the experiment, must be considered. Although these points have been satisfactorily controlled in most of the investigations a careful analysis of the results suggests that a due consideration has not been given to the factor of age. The greater part of the reported experiments were initiated on young animals during the period in which active formation of glomeruli (Arataki, 1926, *b*) is taking place and it seems logical to assume that an animal may have greater powers of adaptation during this early period.

The fact that most of the observations mentioned in the literature reporting no pathologic renal changes were initiated on young rats, favors the above suggestion that such animals are less susceptible to the injurious effect of the ingestion of high concentration of protein. There are, however, experiments reporting negative results with a small number of adult animals (Miller, 1925, and Jackson and Riggs, 1926). In general the positive reports of the production of renal lesions by feeding of excessive amounts of protein have one or both of two serious objections, namely, the choice of the experimental animal or the inadequate and ill balanced diets in regard to the important accessory food requirements. Both of these factors are controlled in the experiments reported by Polvogt, McCollum and Simmonds (1923) who mention the production of lesions by feeding high protein diets. In their experiments young animals were placed on the diet in question for periods ranging from 129 to 485 days. An analysis of their protocols shows that the lesion described in the shorter periods consists in the variable factors of congestion with cellular detritus and serum in the tubules while the really significant lesions, the glomerular adhesions, are described only in animals that have been on the high protein diet for 400 days or more. Similarly, in the experiments reported by Osborne, Mendel, Park and Winternitz (1927), all animals having "severe" lesions were more than 400 days old. These findings are confirmatory evidence of the truth of our suggestion that the kidneys of young animals during the formative stage may be less susceptible to the irritating factor in high protein diets than adult animals.

Although the exact mechanism whereby structural lesions develop in animals ingesting excessive amounts of protein is not clear, two probable factors are suggested: either the changes are associated with the increased burden upon the kidney or, what is more probable, they result from the elimination of some injurious product of protein metabolism.

In the present studies such factors may include the high acidity of the urine, the phosphates, the urea and possibly amino acids. Addis, MacKay and MacKay (1926) have shown that no anatomic lesions are produced in a normal white rat with two kidneys by feeding diets containing an excessive acid or phosphate content. Neither Hinman (1923), Osborne, Mendel, Park and Winternitz (1927) nor MacKay,

MacKay and Addis (1927) were able to demonstrate consistent renal enlargement when quantities of urea equivalent to the protein in diet were given. These observations suggest that increased work is not the only important factor but they do not eliminate the possibility that the persistent effect of unusually large quantities of urea may result in structural damage to the kidney under the conditions of the present experiments. On the other hand Newburgh and Marsh *et al.* (1925) have described renal lesions after the intravenous administration of certain amino acids (lysine, histidine, tyrosine, tryptophane and cystine). The proliferative character of the epithelial changes suggests the persistent action of a mild irritant producing no extensive necrosis but by continual or frequently repeated slight injuries finally resulting in the lesions described above. Further investigation is necessary to determine the specific factor involved in the production of the lesions under the experimental conditions outlined in the present study.

SUMMARY.

The effects of the ingestion of diets containing different concentrations of protein on the remaining kidney in adult white rats after a unilateral nephrectomy has been studied.

In the animals on the high protein diet (85 per cent casein), actual glomerular and tubular lesions were observed in the kidneys of animals maintained for 90, 120 and 150 days after nephrectomy.

In the animals on the standard ration, 18 per cent casein, no significant renal lesions were observed within the experimental period.

Spontaneous focal lesions in the kidneys of rats maintained on Sherman's diets "A" and "B" were inconspicuous at the age of 350 days but became progressively more frequent and were commonly observed after 500 days. The animals on the high protein and standard rations were all under 350 days old at the completion of the experiment.

It is suggested that the age factor is of importance in that young animals may have greater powers of adaptation in withstanding the injurious effect of high protein rations.

The animals on the high protein ration excreted definitely larger quantities of protein in the urine, and showed a higher incidence of casts in periods roughly corresponding to those in which anatomic lesions were observed than did the rats on the standard diet.

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EXPLANATION OF PLATES.

PLATE 4.

FIG. 1. Rat 584. High protein diet for 120 days following a right nephrectomy. There is a small fibrous adhesion between the tuft and Bowman's capsule. × about 305.

FIG. 2. Rat 564. High protein diet 150 days following a right nephrectomy. There is marked proliferation of the epithelium lining Bowman's capsule with small adhesions between the tuft and capsule. There is a focal accumulation of small round cells adjacent to the glomerulus. × about 305.

FIG. 3. Rat 562. High protein diet for 120 days following a right nephrectomy. There is a large fibrous adhesion between the tuft and capsule involving approximately one-half of the circumference of the glomerulus. × about 305.

PLATE 5.

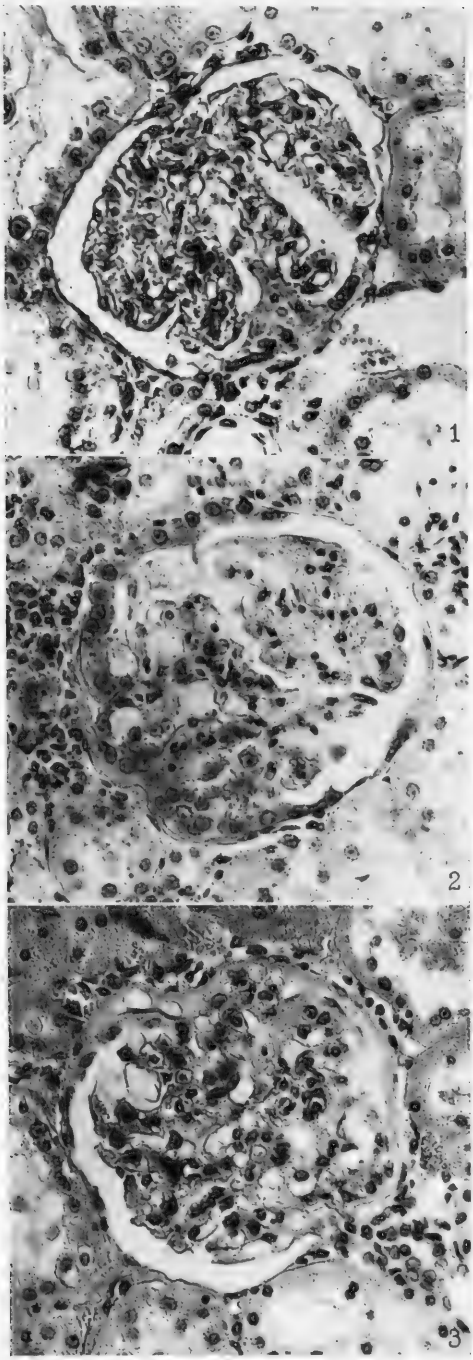
FIG. 4. Rat 674. High protein diet for 150 days following a right nephrectomy. There is a large fibrous adhesion between the tuft and capsule with fibrosis of approximately one-half of the tuft. × about 300.

FIG. 5. Rate 564. High protein diet for 150 days following a right nephrectomy. The figure shows two dilated tubules lined with newly formed epithelium. A mitotic figure is seen in the center of the figure. × about 360.

FIG. 6. *A*, Rat 667; *B*, Rat 674. High protein diet for 150 days following a right nephrectomy.

A—The tubules are moderately dilated. The lining epithelium is cubical with clear and vesicular nuclei. The tubules are widely separated due to an increase in the interstitial tissue. × about 300.

B—The tubules are markedly dilated. The lining epithelium is flat. There is no evidence of cellular activity. × about 300.



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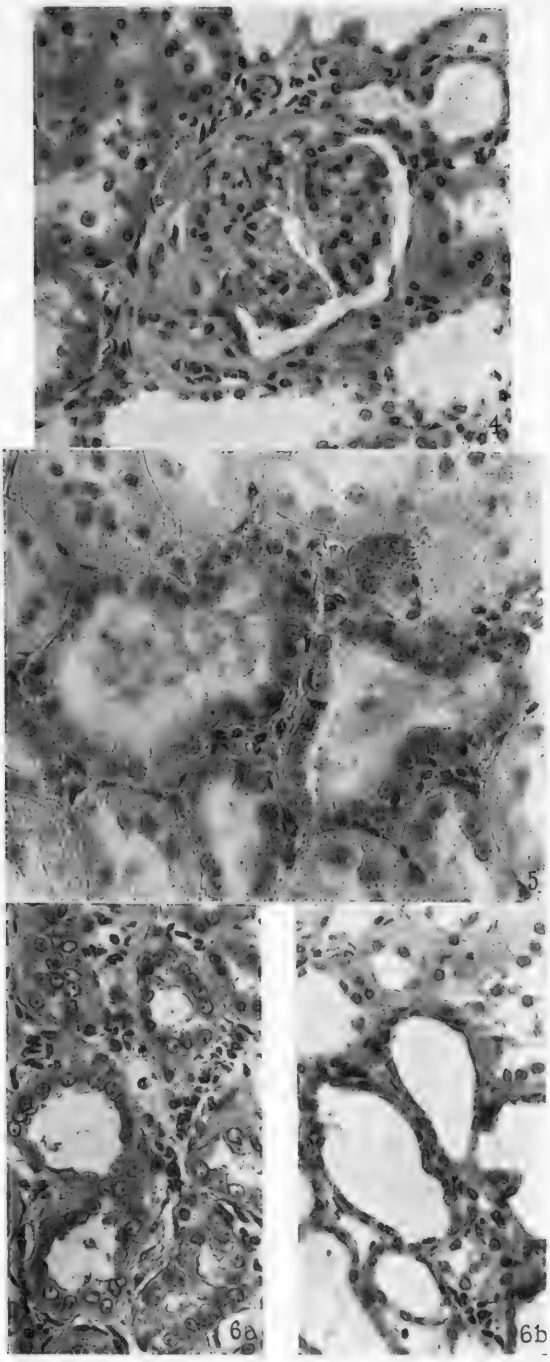

(Moise and Smith: Diet and tissue growth. V.)

CELL RESPIRATION STUDIES.

I. A Microspirometer for the Continuous Study of the Oxygen Absorption by Living Cells.*

By BLAKE C. WILBUR, M.D., GENEVA A. DALAND, and JOHN COHEN, M.D.

(From the Medical Service of the Collis P. Huntington Memorial Hospital of Harvard University, Boston.)

(Received for publication, April 4, 1927.)

The study of the ultimate metabolism of living organisms resolves itself into the analysis of the process in individual cells. While certain facts may be learned from the study of unicellular organisms, the specialized cells of the more complicated structures of mammals present additional problems. For the study of the respiration of such cells, numerous methods have been devised. Some depend on the oxidation or reduction of chemical agents, and others depend on the principle of gas analysis. The criticisms of most of the published methods are that the cells are exposed to abnormal conditions of environment, temperature, and osmotic pressure, and that the quantitative methods do not permit the continuous study of the same cells over long periods of time. It is possible to note the disappearance of oxygen in whole blood by determining the oxygen content at the beginning and at the end of a definite period, but in the actual chemical analysis the cells are killed. In such methods the number of observations is limited and the composition and identity of the cells are naturally not homogeneous.

To obviate these criticisms, an apparatus was designed to allow quantitative measurements of the volume of oxygen over a sample of cells. In the experiments with this apparatus seeds, pieces of tissue, and blood corpuscles were used, without disturbing their vital processes. The living structures during the time of the experiment

* The expenses of this investigation have been defrayed in part by a grant from the Proctor Fund of the Harvard Medical School for the study of chronic diseases.

were kept at a uniform temperature and in intimate relation with the respiratory medium.

The principle on which this microspirometer works is similar to that of most forms of closed space respiration apparatus. It has the advantage of this type of apparatus in that very accurate determination of the oxygen consumption can be obtained with comparative ease over short periods of time. It may be used for experiments involving a composition of gases differing from the atmosphere. The variations in the volume of the oxygen in the respiratory chamber may be measured during a given interval of time if all the carbon dioxide produced is absorbed by a solution of potassium hydroxide. The difference in the volume at the beginning and at the end of a given period, when the temperature and pressure are kept constant, is a measure of the amount of oxygen absorbed. Readings of the volume of oxygen used may be made at regular intervals during the course of the experiment.

Among the several forms of closed space microspirometers described by others, the differential microspirometer for the direct measurement of the consumption of oxygen designed by Barcroft and Roberts (1) and later modified by Krogh (2), and by Warburg (3), and also the one used by Winterstein (4), present certain technical problems, when blood is used, which make the results difficult to interpret. The apparatus designed by Koehler (5), though easy to manipulate, is not practical for small amounts of blood. Various forms of apparatus such as the Van Slyke (6) and the Haldane (7) measure changes in oxygen content, but the cells are killed in the process.

Such factors as the difference in vapor pressure between the material examined and the absorbing fluid, the difference in vapor tensions over the leveling fluids in the differential capillary tubes, the incomplete absorption of carbon dioxide, insufficient shaking or stirring, and the like, are sources of error which modify appreciably the results. The apparatus designed in this laboratory appears to eliminate these sources of error.

The Apparatus.

A diagram of the microspirometer described below is shown in Fig. 1. It is constructed of Pyrex glass.

For convenience the whole apparatus is mounted on a board and placed on a stand so that it can be put in a water bath with the level of the water just above the transverse connections and stop-cocks.

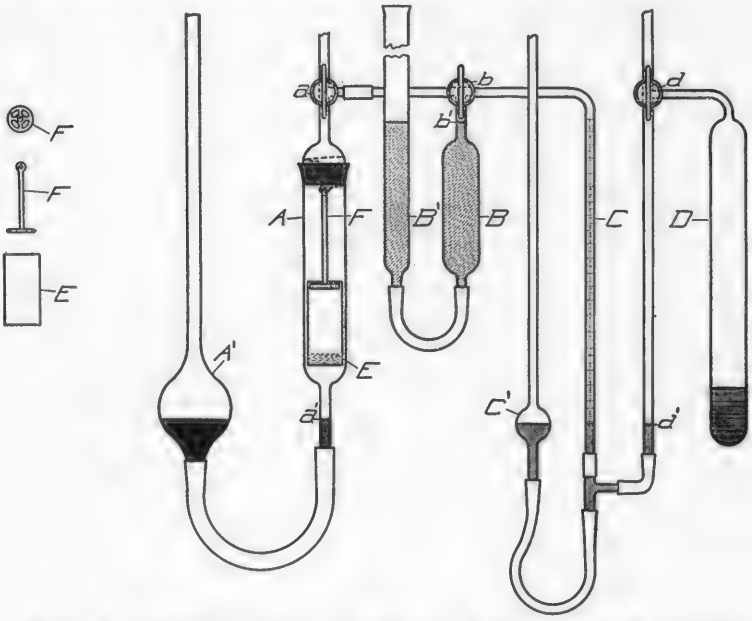

FIG. 1. A, metabolism chamber with reservoir A' for mercury. B, absorption chamber with reservoir B' for potassium hydroxide. C, manometer tube with reservoir C' for bile solution. D, compensation tube. E, cup to hold material for study. F, stirring rod. At the left the face view shows the perforated disk. a, b, and d represent the three-way stop-cocks which are attached to the chambers with corresponding letters. a', b', and d' represent the level to which the solutions are raised when readings are taken.

The microspirometer consists essentially of four parts:

1. The respiration or metabolism chamber A which holds a small cup E into which the material to be studied is placed.

The outside measurement of the metabolism chamber A is 19 × 3.9 cm. The bottom narrows down leading through a capillary tube into a rubber hose connected with the mercury reservoir A', the diameter of which is 7 cm. The top of

the metabolism chamber is closed with a tightly fitting ground glass stopper, on which there is a three-way stop-cock leading from this chamber to the outside and to the absorption chamber. Inside the stopper there may be placed a small brass spring to suspend the stirring rod *F*. The stirring rod consists of a perforated glass disk, 2.3 cm. in diameter, on the end of a glass rod 6 cm. long. The cup *E* in the metabolism chamber is 6.5 cm. deep and 3.3 cm. in diameter.

2. The absorption chamber *B* which contains the potassium hydroxide solution to absorb the carbon dioxide in the air driven over from the metabolism chamber.

The absorption chamber measures 15 × 3.5 cm. Its outlet to the reservoir *B'* is 0.5 cm. in diameter and the reservoir *B'* is 45 × 25 cm.

The concentration of the potassium hydroxide used is 0.96 N.

3. The manometer tube *C* which indicates the change of volume resulting from the consumption of oxygen by the material studied.

The manometer tube has a bore of about 0.3 cm. and it is 30 cm. long. It is graduated into tenths of a cm. and has a volume of 2.2 cc. Calibration shows each cm. to equal 0.0735 cc. at 37.5°C. The reservoir *C'* is 3.5 cm. in diameter.

A solution of bile salt containing 23 gm. sodium chloride and 5 gm. sodium taurocholate in 500 cc. of water (8) is used in the manometer. This solution has the advantage of dissolving fat, and therefore runs evenly in the capillary tubes. It does not absorb oxygen or carbon dioxide to a significant extent.

4. The compensating tube *D*, which is attached to the manometer to correct for changes in temperature and barometric pressure after the apparatus is closed.

This chamber has the same volume (148 cc.) as the metabolism chamber *A*. For each cc. of material added to the latter an equal volume of water should be placed in the compensating chamber *D*.

Procedure.

The reservoirs attached to the apparatus are filled with the proper fluids. Enough mercury is placed in the reservoir *A'* attached to the metabolism chamber *A*, to raise the cup *E* to the top of the chamber when the reservoir is elevated. 2 or 3 drops of the bile solution are placed on top of the mercury in the metabolizing chamber *A* to prevent the mercury from adhering to the sides and thus interfering with the readings.

The potassium hydroxide solution is placed in the reservoir B' and allowed to fill the absorption chamber B to the mark b'. The manometer C and its reservoir C' are partly filled with the bile solution.

To make the apparatus absolutely air-tight the stop-cocks must be cleaned and lubricated with a suitable stop-cock grease (*e.g.* Ramsay) for each experiment.

When the apparatus is ready, the material to be studied is placed in the cup inside the metabolism chamber A. The ground glass stopper is well lubricated with stop-cock grease and sealed.

The entire apparatus is then placed in a water bath maintained at 37.5°C., or any other desired temperature. In the water bath used it was found possible to maintain a temperature of 37.5°C. with a maximum variation of 0.2°. The temperature may be observed every 5 to 15 minutes on a Beckmann metastatic thermometer, during the course of each experiment (1 to 5 hours). The water bath is heated with electric light bulbs, controlled by a mercury level thermostat. The water is kept in circulation by a motor-driven stirring apparatus. An hour is allowed for the apparatus to acquire the temperature of the bath. The microspirometer is left in the water bath throughout the experiment and readings may be made through a glass window in the side of the water bath.

When a fluid substance, as blood, is to be tested it may be added, after the apparatus is warmed, through a long, hollow needle, inserted through the stop-cock a.

Before observations are commenced all of the stop-cocks are opened to the air, then the mercury is leveled to the mark a', the potassium hydroxide is leveled to the mark b', and the bile solution is brought to the mark d' of the compensating tube. The manometer reading is noted and then the apparatus is closed to the air with the fluids at these levels. Stop-cock b is then closed so that the absorption chamber connects only with the metabolism chamber, which is closed off from the outside by stop-cock a. The compensating tube is closed so that it connects only with the manometer tube.

When blood or a fluid is studied it should be stirred constantly. To do so the mercury reservoir is raised and lowered slowly but continuously, so that the perforated disk suspended in the metabolism chamber agitates the fluid as it rises and falls. If agitation is unneces-

sary, as with solid substances, then at any desired period of time the mercury reservoir is raised and lowered several times to drive the gases over into the absorption chamber.

To make a reading of the new volume, the mercury is lowered slowly until the potassium hydroxide rises to the mark b'. When the solution is at this level, stop-cock b is turned so that the absorption chamber is closed off and the metabolism chamber is connected with the manometer. The mercury is now set at the mark a'. To make the reading, the bile solution must be brought to the mark d' in the compensating tube, and then the level in the manometer recorded. Leveling the bile solution at the mark d' corrects any changes in volume due to barometric and temperature variations. The diminution of volume recorded after this procedure is the result of the consumption of oxygen by the material studied, as the carbon dioxide produced has been absorbed. Any carbon dioxide remaining in solution does not influence the results as it is oxygen consumption that is desired. Any oxygen that may be in solution in the material studied and in the potassium hydroxide remains practically constant throughout the experiment, because the change in the tension is too small to affect measurably the amount of the dissolved gas. If a reading is taken before absorption of the carbon dioxide the quantity produced may be determined by reading the volume before stirring. This will give the difference between the oxygen consumed and the carbon dioxide produced, and after the absorption of carbon dioxide the remaining volume indicates the amount of oxygen consumed.

The apparatus must be calibrated by liberating or withdrawing known volumes of gas and measuring the change in level of the bile column in the manometer tube. The gas may be liberated chemically, or air or carbon dioxide may be added from a graduated pipette or syringe through stop-cock a. In experiments of this type the observed readings were what they should have been and well within the limits of experimental error. In the microspirometer described above, the volume change in the manometer tube multiplied by 3.2 gives the volume change in the metabolism chamber.

The error in reading the level of the bile solution in the manometer tube is 0.5 mm., which is equivalent to 0.00367 cc. of gas in the manometer or 0.01174 cc. in the volume of the metabolism chamber. The

bore of the capillary tubes, in which the mercury of the metabolizing chamber at a' and the potassium hydroxide at b' are leveled, is practically the same as that of the manometer, so that the error of reading these levels is no greater. When blood is placed in the microspirometer and the stop-cocks closed to the outer air, the gas in the chamber slowly increases in volume, during the course of 5 to 30 minutes. A decrease due to oxygen absorption by the cells becomes apparent after the maximum increase has been attained and equilibrium has been reached. The preliminary increase in volume may be the result of several factors. The blood cools slightly when drawn and there is an increase in the temperature when it is put into the apparatus. This may result in changed vapor tension as well as changed solubility of dissolved gases. It is also possible that, under the conditions of the experiment, methemoglobin may be formed, with liberation of oxygen. Spectroscopic examination of the blood after the observations had been completed were made by Dr. Mirsky and Dr. Anson, but the amount of methemoglobin, if present, was too small to be noted by the spectroscope.

The procedure used for studying blood has been as follows:

8 cc. of human blood are withdrawn from the median vein of the arm into a syringe containing a solution of heparin[1] as an anticoagulant. The syringe is gently agitated to thoroughly mix the heparin with the blood. If it is desired, the blood may be saturated with oxygen. 5 cc. are then put into the cup E of the warmed microspirometer, through a hollow needle, inserted in stop-cock a. The fluids are brought to a level at their respective marks and the apparatus closed to the outside air. Stirring is commenced at once and continued throughout the experiment in order to keep the blood oxygenated and continually in motion. Readings are taken at about 10 minute intervals. The rest of the blood may be used for quantitative morphological studies.

When isolated corpuscles are used instead of whole blood, the procedure is the same. The blood is drawn into a syringe containing heparin and then centrifuged. The plasma and white cells are removed separately. Enough of the plasma is then added to the cells to make up the desired volume. Quantitative studies of the cells in the resulting suspension may be made.

[1] 0.05 cc. of a 2 per cent solution of heparin is used for 5 cc. of normal blood. Twice as much is used when working with leucemic blood, with a high white blood cell count.

One may readily observe the consumption of oxygen by many forms of living material. At the suggestion of Dr. Alfred C. Redfield, sprouting radish and lupin seeds were used for this purpose and the rate of oxygen consumption was measured at frequent intervals over a period of 2 days. The results show a uniform rate of oxygen consumption with the growth of the seeds.

The amount of oxygen used by normal or tumor tissues is readily measurable by this spirometer.

In working with an apparatus of this type it should be borne in mind that the actual rate is a function of the shape and size of the vessel in which the material is exposed and the rate at which it is stirred. If the process which liberates or consumes gas proceeds at a uniform rate, and the rate of stirring (and, therefore, the rate of exposure of carbon dioxide, when formed, to the potassium hydroxide) is uniform, the rate per hour is the same at different times. If, under these conditions, the rate per hour differs from time to time, it is suggestive that the process does not proceed at a uniform speed. Data as to rate are comparable only when the stirring is uniform. The "lag" of the mercury in the capillary tube acts as a governor, controlling the speed of the stirring, and this helps to keep it uniform.

Comparative studies of the oxygen consumption of normal and pathological bloods are of value if they are carried out under similar technical conditions.

CONCLUSION.

A description is given of a closed space respiration apparatus which can be used to determine the amount of gas used or liberated by living blood or tissue cells, or chemical substances. Continuous observations can be made and repeated measurements recorded without interrupting the vital processes or destroying the cells.

Studies of the oxygen consumption by whole blood in normal individuals and in patients with leucocytosis and myelogenous leucemia, as well as by white cells suspended in plasma, will be reported in subsequent papers.

It is a pleasure to acknowledge our indebtedness to Dr. George R. Minot, Dr. Thomas E. Buckman, Dr. Cyrus C. Sturgis, and Dr.

Raphael Isaacs for the valuable criticisms and suggestions which have made this work possible.

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CELL RESPIRATION STUDIES.

II. A Comparative Study of the Oxygen Consumption of Blood from Normal Individuals and Patients with Increased Leucocyte Counts (Sepsis; Chronic Myelogenous Leucemia).

By GENEVA A. DALAND and RAPHAEL ISAACS, M.D.

(*From the Medical Service of the Collis P. Huntington Memorial Hospital of Harvard University, Boston.*)

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The observation by Warburg (1) that the amount of oxygen used by a slice of Flexner-Jobling rat carcinoma was less than that absorbed by such normal tissues as liver or kidney, has opened the way for the further study of the metabolism of tumor and other rapidly growing cells. The immature white blood cells of myelogenous leucemia resemble somewhat those of the malignant neoplasms in their growth rate and behavior, and response to exposures to Roentgen rays. They serve as excellent material for the study of oxygen consumption because of their availability and the ease with which they can be manipulated *in vitro*.

Patients with leucemia frequently have an elevated basal metabolic rate, particularly when the blood contains many immature cells (Riddle and Sturgis (2)). The possibility of a causal relationship between the rate of oxygen absorption by the patient and the degree of maturation of the blood cells presents a problem toward the solution of which this study of cell respiration in the microspirometer offers some data.

The studies reported here include observations on the blood of normal individuals, a patient with a leucocytosis from sepsis, and patients with chronic myelogenous leucemia in various stages of the disease.

Methods.

The details of the use of the special microspirometer have been reported elsewhere (3). The method of Lundsgaard and Möller (4) was used in the determination of the oxygen content of the blood in the Van Slyke blood gas apparatus. Blood from the arm veins was used and the enumeration of the cells was made by the standard methods. In making the differential leucocyte counts, 500 to 1000 white blood cells were studied.

DATA.

Normal Blood.

When the venous blood from a normal individual is transferred directly to the microspirometer part of the oxygen is used to convert the reduced hemoglobin to oxyhemoglobin. The amount used in excess of this represents the oxygen used for metabolism by the cells.

The oxygen content of a specimen of blood can be measured at the beginning and end of an experiment and the amount of oxygen necessary for saturation of the hemoglobin can also be determined by use of the Van Slyke blood gas apparatus. The microspirometer, however, shows the total amount of oxygen used during a similar period of time.

The results are reported of sixteen observations in which the hemoglobin was unsaturated and six cases in which the hemoglobin was "saturated" before placing in the chamber.

In sixteen specimens of normal blood which were transferred directly to the microspirometer without being exposed to the air, the average amount of oxygen used was 6.66 cc. per 100 cc. of blood. An example of such an experiment in which the blood was not previously saturated with oxygen is shown in Table I.

In the experiment recorded in Table I the difference between the oxygen content at the beginning of the observations and at the completion of the experiment, as measured by the Van Slyke apparatus, was 5.49 cc. The degree of unsaturation of the blood at the beginning was 6.24 per cent, so that the blood became practically saturated in the microspirometer.

The amount used in 1 hour was 6.72 cc. of oxygen per 100 cc. of

blood. This included the 6.24 cc. to saturate the hemoglobin, so that the actual consumption in this case was 0.48 cc. This is such a small amount that it is hardly significant, as it is but little over twice the possible error which arises in reading the manometer. The results in fifteen other experiments with normal blood under similar conditions were entirely comparable.

In six specimens of normal blood which were "saturated" with oxygen outside of the apparatus the oxygen consumption averaged

TABLE I.

Rate of Oxygen Consumption by Blood of Normal Individual.

Time	Amount of oxygen used by 100 cc. venous blood	Time	Amount of oxygen used by 100 cc. venous blood
min.	*cc.*	*min.*	*cc.*
0	0	80	8.89
5	0.47	90	10.02
20	2.07	110	11.42
30	3.10	150	11.88
40	5.55	160	14.49
60	6.72	165	15.33
70	8.28	175	15.33

Oxygen content at beginning of observations..... 17.55 cc. per 100 cc. of blood
" " " end of observations..... 23.04 " " 100 " " "
Oxygen-combining power..... 23.79 " " 100 " " "

The microspirometer was in the water bath at 37.5°C. for 1½ hours before the blood was placed in the metabolism chamber. 15 minutes elapsed from the time that the blood was drawn to the first reading on the manometer.

0.055 cc. per 100 cc. of blood. The error in reading the meniscus of the bile tube may represent 0.235 cc. so that the oxygen absorption of normal blood, as determined by this microspirometer, is zero within the limits of experimental error.

Polymorphonuclear Neutrophil Leucocytosis.

The simplest change in blood that may affect its metabolism is an increase in the number of white blood corpuscles due to leucocytosis. In blood with a high leucocyte count, the absorption of oxygen be-

came appreciable under the conditions of these experiments. Table II shows data concerning the oxygen absorption by this blood from a patient with an infected tumor growth. The white blood cell count was 24,000 per c.mm. and 85.6 per cent of the cells were mature polymorphonuclear leucocytes. The blood was saturated with oxygen for 5 minutes in a large flask. The preliminary increase in volume of the respiration chamber gases, noted in all the experiments and referred to in the preceding paper (3), continued for 60 minutes after the microspirometer was closed. The first decrease in volume was recorded 67 minutes after the blood was drawn.

TABLE II.

Rate of Oxygen Consumption by Blood with a Polymorphonuclear Neutrophil Leucocytosis.

(White blood cells 24,000 per c.mm. 20,000 polymorphonuclear neutrophils.)

Time	Amount of oxygen used per 100 cc. oxygenated blood	Time	Amount of oxygen used per 100 cc. oxygenated blood
min.	*cc.*	*min.*	*cc.*
0	0.0	30	0.71
10	0.25	50	1.18
20	0.47	60*	1.41

* During the next 50 minutes there was no more oxygen absorption as indicated by identical readings of the level of the bile in the manometer.

Two sets of observations on the blood of the same patient on different days gave the following results: When the absolute white blood cell count was 22,250 per c.mm. (19,180 polymorphonuclear neutrophils) the oxygen consumption was 2.75 cc. per hour per 100 cc. of blood. When the count was 18,700 (15,800 polymorphonuclear neutrophils), the oxygen consumption was 1.73 cc. per hour per 100 cc. of blood.

The oxygen content at the end of the experiment was less than that at the beginning, and the hemoglobin was 4.73 per cent unsaturated, even though it had been exposed to oxygen throughout the observations. This was different from the normal bloods and was comparable to the condition in the leucemic bloods described later.

Chronic Myelogenous Leucemia.

In blood from patients with chronic myelogenous leucemia, where many of the cells are of an immature and often atypical sort, the rate of oxygen absorption is easily measurable. The observations recorded in Table III illustrate the rate and amount of oxygen absorption in the blood of a woman who had had leucemia for $2\frac{1}{2}$ years.

TABLE III.

Oxygen Consumption by Blood of a Patient (No. 5) with Chronic Myelogenous Leucemia.

Time	Amount of oxygen used per 100 cc. of oxygenated blood	Time	Amount of oxygen used per 100 cc. of oxygenated blood
min.	*cc.*	*min.*	*cc.*
0	0.0	140	18.34
10	0.47	150	19.28
20	1.88	160	20.22
30	3.29	170	21.16
40	4.23	180	23.52
50	5.65	190	25.86
60	6.59	200	27.75
75	7.99	210	30.15
90	10.35	220	32.44
100	12.23	230	34.34
110	13.64	240	34.34
120	15.52	250	36.68
130	17.40	260	37.63

Oxygen content at beginning of observations..... 13.55 cc. per 100 cc. of blood
" " " end of observations..... 2.80 " " 100 " " "
Oxygen-combining power..... 13.55 " " 100 " " "

At the time the tests were made the white blood cell count of the venous blood was 176,000 per c.mm. and only 19.4 per cent of the cells were mature. The rest were grossly immature, chiefly myeloblasts.

The difference between the oxygen content at the beginning of the observations and at the completion of the experiment, as measured by the Van Slyke apparatus, was 10.75 cc. Although the blood was saturated at the beginning, it was very far from saturated at the

end of the observations. The amount of oxygen used by the same sample of blood in the microspirometer during this period was 37.63 cc.

Another portion of the same specimen of blood was kept under oil in an incubator, at 37.5°C. and the oxygen content was deter-

TABLE IV.

Oxygen Consumption by Blood in Ten Cases of Chronic Myelogenous Leucemia.

Patient No.	White blood cells per c.mm.	P.M.N.* per c.mm.	Immature cells per c.mm.	Red blood cells per c.mm.	Hemoglobin	Amount of oxygen consumed per hr. per			
						100 cc. whole blood	1000 W.B.C.	1000 mature P.M.N.	1000 immature leucocytes
							$\times 10^{-6}$	$\times 10^{-6}$	$\times 10^{-6}$
				millions	*per cent*	*cc.*	*cc.*	*cc.*	*cc.*
1	37,000	23,700	5,900	2.8	53	4.48	1.210	2.314	7.593
2	52,000	21,600	28,500	3.0	55	3.84	0.730	1.780	1.347
3	122,000	38,300	79,300	3.2	73	9.72	0.796	2.537	1.225
4	123,000	47,900	67,700	3.1	68	12.67	1.036	2.641	1.874
5	176,000	34,100	140,800	3.1	64	7.30	0.414	2.138	0.518
6	454,000	90,800	345,000	3.0	59	18.56	0.408	2.044	0.538
Average						9.42	0.765	2.420	2.182
7	71,000	29,100	38,300	2.2	73	6.14	0.864	2.109	1.603
8	80,000	30,200	45,600	4.0		6.46	0.807	2.136	1.416
9	111,000	38,000	54,800	4.1	72	5.89	0.526	1.549	1.076
10	251,000	20,800	230,200	2.9	35	8.77	0.349	4.200	0.381
Average						6.815	0.636	2.468	1.119

* The absolute number of mature polymorphonuclear neutrophils is contrasted with the absolute number of immature cells which include myelocytes of all stages and myeloblasts. The number of eosinophilic and basophilic polymorphonuclear cells is not included. The number of lymphocytes, which is comparatively small in most leucemic bloods with high white counts, is disregarded in this comparative study.

mined at intervals by Dr. Arlie V. Bock. Practically all the oxyhemoglobin was reduced in 3 hours. The oxygen-combining power at the start was 13.55 cc. per 100 cc. of blood, and at the end was 0.04 cc. per 100 cc. of blood.

The data of the first six cases of chronic myelogenous leucemia

given in Table IV are better for comparative purposes than the other four cases because the red blood counts (2.8 to 3.2 millions per c.mm.) and the hemoglobin percentages (53 to 68, Sahli) were similar enough to be practically the same. The color indexes of these six patients were: 0.94; 0.91; 1.1; 1.09; 1.03; and 0.98 (average 1.008) while those of the others were 1.6; 0.87; and 0.61. The influence of the number of red blood corpuscles and the per cent of hemoglobin thus did not enter significantly into variations in oxygen absorption by the first six cases enumerated so that it is proper to attribute variations in metabolic activity especially to the leucocytes.

DISCUSSION.

When blood from a normal individual is drawn from a vein and transferred directly to the microspirometer, part of the oxygen absorbed is used to convert the reduced hemoglobin to oxyhemoglobin. The amount used in excess of this represents, to a great extent, the oxygen absorbed by the leucocytes. The reticulated red blood cells (young cells) apparently have the power to absorb oxygen (Morawitz (5), Morawitz and Itami (6), Harrop (7), and Denecke (8)). In the cases of leucemia with a high white blood cell count they formed but a minor fraction of the total number of metabolizing cells, so that the amount of oxygen that they used was but a small part of the total. The part played by the blood platelets in the consumption of oxygen is not known.

In normal blood, when the hemoglobin is saturated, the oxygen consumption is less than in blood in which the number of leucocytes is high. Furthermore, the process of utilization of oxygen lasted longer in the leucemic blood and that from the patient with leucocytosis than in a normal blood. The readings on the manometer tube usually became constant after $1\frac{1}{2}$ hours when normal blood was being studied, while a constant progressive change was noted in the bloods with high white counts from $1\frac{1}{2}$ to over 10 hours, and possibly longer. Leucemic blood used from 3.7 cc. to 70.5 cc. of oxygen per 100 cc. of blood during the course of the period of observation (*i.e.* until absorption stopped or the experiment was discontinued) while there was practically no absorption by normal blood and 1.41 cc. to 1.76 cc. by the blood of the patient with leucocytosis, when absorption was complete.

When the different leucemic bloods and the one with simple leucocytosis are compared, and an effort is made to correlate the amount of oxygen used per hour with the total white blood count, the total number of immature white blood cells, the total red blood cell count, the hemoglobin percentage, and the total number of mature polymorphonuclear neutrophils, the highest degree of correlation is found with the last named factor. This is shown in Chart 1. In

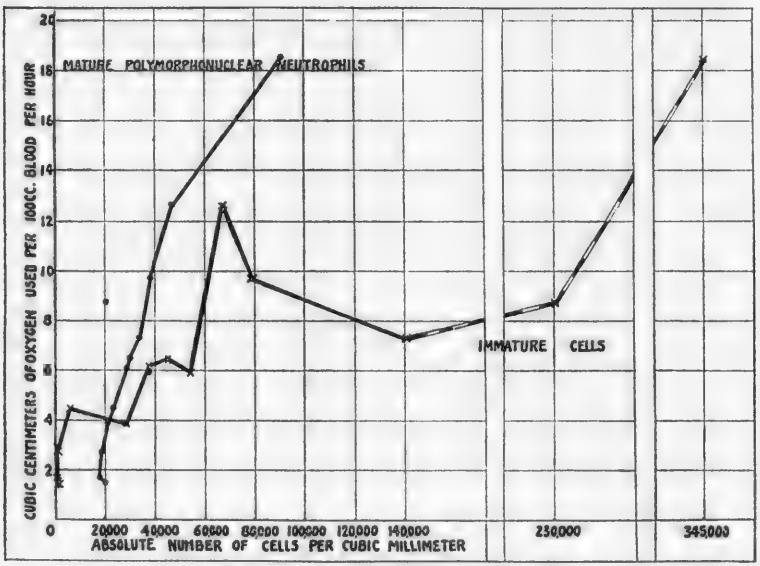

CHART 1. Rate of oxygen consumption by 100 cc. of blood compared with the absolute number of mature polymorphonuclear neutrophils and with the number of immature cells in sepsis and in chronic myelogenous leucemia.

Table IV it will be noted that the individual observations vary from the average the least when it is assumed that the bulk of the oxygen absorbed is taken up by the mature polymorphonuclear neutrophils, and the variation from the average is much greater when the total white blood cell count or the number of immature cells is used for comparison. The adult cells appear to use more oxygen than the immature ones under the conditions of these experiments as shown

in Table IV. In Patient 5 the number of immature cells was 176,000 per c.mm., whereas in Patient 4 the number of immature cells was 67,650 per c.mm. In the former the rate of oxygen absorption, under the conditions of this microspirometer, was only 7.30 cc. per hour per 100 cc. of blood whereas in the latter the consumption was 12.67 cc. per hour. Evidently the immature cells used less oxygen than a corresponding number of mature cells. Whether this is true of the cells in the body or not is a matter for additional study. The immature cells may be more susceptible to changes in their environment than the adult cells, and therefore show a lower rate of metabolism *in vitro*.

If the rate of metabolism of the immature white blood cells is low, as compared with that for the adult cells, the former resemble tumor cells in this respect more than embryonic or young tissue cells. The immature leucocytes behaved as did Warburg's tumor tissue (1) when exposed to the conditions of a microspirometer. These observations also make it seem improbable that there is a direct relation between the oxygen metabolism of the immature blood cells in the peripheral circulation and the increased basal metabolic rate of the leucemic patients. The hemoglobin of normal blood, as noted after it has been for a long period of time in the microspirometer, remains saturated with oxygen. This is sharply in contrast to what happened under similar circumstances to the leucemic blood, for it was found that its hemoglobin was no longer saturated with oxygen, although it was exposed to the air of the respiration chamber and kept in motion. When such blood was taken from the apparatus and exposed to the air of the room it was found that it did not take up oxygen to the full capacity of the hemoglobin present, so that in some cases it was as much as 4.78 volumes per cent desaturated. As this was a fairly constant observation for leucemic blood when the white blood cell count was high, and not characteristic of normal blood as noted by us and by others (Harrop (7)), it is thought that there may have been some factor which changed part of the hemoglobin. However, there was no definite evidence of this and spectroscopic examination failed to show the presence of methemoglobin.

The carbon dioxide was absorbed in all these experiments as fast as it was produced. Evidently some "fixed" acid, probably lactic,

was formed as in those experiments on leucemic blood in which the carbon dioxide-combining power of the plasma was measured before and after the observations. It was found that the carbon dioxide-combining power was reduced in the leucemic bloods to practically nothing as the figure obtained was so small that it was within the limits of possible error of the method of determination.

There were no gross changes noted in general morphologic characters of the cells after they had been in the microspirometer for the period of the experiments. They appeared normal in all respects when studied with supravital stains or examined in films colored with Wright's stain. The oxidase reaction was the same or possibly more intensely positive at the end than at the beginning of the experiment.

SUMMARY AND CONCLUSIONS.

1. The oxygen consumption of blood of normal individuals, when the hemoglobin is saturated with oxygen, is practically zero within the limits of experimental error of the microspirometer used.

2. The oxygen consumed in a microspirometer by the blood of patients with chronic myelogenous leucemia with a high white blood cell count, and of one with leucocytosis from sepsis, was proportional to the number of adult polymorphonuclear neutrophils in the blood.

3. No correlation could be made between the rate of oxygen absorption and the total number of white blood cells in the blood, or the total number of immature cells, or the number of red blood cells, or the amount of oxyhemoglobin.

4. The blood of patients with chronic myelogenous leucemia continued to use oxygen in the microspirometer longer than that of normal individuals, and the hemoglobin, in the leucemic bloods, became desaturated even though exposed to air.

5. In blood in which the bulk of the cells were immature and the mature cells few, the oxygen consumption was lower than in blood in which the mature cells predominated. The rate of oxygen consumption of the immature cells was relatively low as compared to the mature.

6. The slower rate of oxygen absorption by the immature leucocytes in chronic myelogenous leucemia as compared to the mature cells, places them, in accord with Warburg's reports, in the class of

the malignant tissues in this respect rather than in the group of young or embryonic cells.

Throughout the course of this work we have received advice and criticism from various associates. We wish to express our appreciation for their assistance, especially to the following: Dr. George R. Minot, Dr. Lawrence J. Henderson, Dr. Thorne M. Carpenter, Dr. James H. Means, and Dr. Blake C. Wilbur.

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EFFECT OF HOST IMMUNITY TO A FILTERABLE VIRUS (VIRUS III) ON THE GROWTH AND MALIGNANCY OF A TRANSPLANTABLE RABBIT NEOPLASM.

By LOUISE PEARCE, M.D., and THOMAS M. RIVERS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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In a previous paper (1) experiments were reported in which it was shown that 2 filterable viruses, Virus III and vaccine virus, grow and survive for unusually long periods in a transplantable malignant neoplasm of the rabbit. Early in the course of this work, it was found that the tumor (2) was infected with Virus III (3) and was being transferred with it, and also that the rabbits so inoculated became immune to Virus III. Since Virus III is indigenous to rabbits and its incidence fairly high judging from the number of immune animals in the normal stocks of these laboratories (15 to 20 per cent on an average), it is impossible to say when the tumor became infected. In any event, the question arose as to whether growth and malignancy of the tumor were affected first, by the presence of the virus, and second, by host immunity to the virus. An opportunity to investigate these problems was afforded by the fact that we were able to obtain the tumor without the virus and to transplant it in the same manner as the stock tumor bearing the virus.

Experiments are reported in the present paper dealing with the effect of the immunity of the host to Virus III on the pathological processes induced by both the virus-bearing and the virus-free tumor strains. A subsequent paper deals with the question of the effect of the virus on the growth and malignancy of the tumor (4).

EXPERIMENTAL.

Materials and Method.

Neoplasm.—The tumor employed in these experiments has been studied extensively ever since its recognition in October, 1920, and its subsequent trans-

plantation, and a number of papers dealing with various aspects of the subject have been published (2). It suffices to state here that the tumor is considered to be of epithelial origin composed of cells allied to those found in the bulb and root sheath of the hair, and that it has been transplanted through successive generations by intratesticular inoculations. This method of inoculation has proven effective, not only from the standpoint of obtaining actively progressing primary tumors, but also for the study of the pathological process as a whole. The manifestations of the disease vary markedly both in individual rabbits of a series and between groups of animals inoculated at one time as compared with those inoculated at another. The growth and fate of the primary tumor, the incidence of metastases, the distribution, number, extent and state of the growths and the mortality rates, both actual and estimated, are among the variable features of the disease which must be taken into account in evaluating the character of the process at any time.

It was found in October, 1924, that all rabbits inoculated with the tumor became immune to Virus III, and by means of rapid passage of emulsions of primary tumors or metastases, the presence of the virus was regularly demonstrated.

Virus III.—While attempting to produce chicken-pox in rabbits, a filterable transmissible agent was discovered (3). This agent produces gross as well as microscopic lesions in the cornea, skin and testicles of rabbits, and an infection with it leads to an immunity against subsequent infections with the same material. For convenience, this agent has been spoken of as Virus III. At first it was considered not unlikely that the virus is the etiological agent of varicella. Further work, however, disclosed the fact that Virus III is indigenous to rabbits and that it is as typical a virus as vaccine virus or the virus of herpes simplex from both of which it can easily be differentiated.

Virus-Free Strain of Tumor.—This strain was obtained from a rabbit inoculated with the stock tumor bearing the virus. The animal died several weeks after inoculation and accidentally lay in a warm room 12 to 18 hours. The primary tumor was removed and inoculated into the testicles of 3 rabbits. Although the development of these transplants was much delayed, growth later occurred and transfers were successfully made Many generations of this strain of the tumor have been studied, and it has been impossible to demonstrate the presence of Virus III by methods which suffice with the stock tumor. Rabbits inoculated with this strain, moreover, do not become refractory to skin infection with Virus III, their sera do not become virucidal and no nuclear inclusions, typical of Virus III reactions, have been found in young tumors. The virus-free state of this strain was controlled from time to time by appropriate tests.

Conduct of Experiments.—The experiments were carried out from February, 1925, to January, 1926. Groups of 10 male rabbits from selected stocks were immunized to Virus III by a single intracutaneous or subcutaneous injection of testicular emulsions containing the virus. At intervals of 20 to 39 days after the injection of Virus III, the rabbits were inoculated in one testicle with 0.3 cc. of a

salt solution emulsion of an actively growing primary tumor. Comparable groups of non-immunized rabbits were inoculated at the same time. Both the stock tumor strain bearing the virus and the virus-free strain of tumor were used. The total number of rabbits employed in the experiments reported in this paper was 129, 49 of which were immunized.

The rabbits were separately caged and fed the same diet of hay, oats and cabbage. Frequent examinations were made, special attention being paid to the general physical condition of the animals, the character and course of the primary tumor and the development of secondary growths in superficial parts of the body.

The experiments were terminated 2 months after inoculation at which time all surviving animals were killed by an injection of air in the marginal ear vein. This period was selected upon the basis of previous experience as being sufficient to include a large proportion of the deaths due to tumor growth, and at the same time, sufficient to allow for the recovery of many rabbits. Rabbits which developed a pronounced cachexia or paralyses during the observation period were killed at that time. Each animal was subjected to postmortem examination, particular attention being given to the state of the primary tumor and to the distribution, number and condition of secondary growths together with an estimation of the degree of organ involvement.

Method of Analysis of Results.—The data obtained from clinical observations and postmortem examinations have been analyzed upon a group basis. The actual deaths from the tumor process have been classified in 2 groups upon the basis of postmortem findings. In one, designated as "malignant," the widespread or significant distribution of tumor was such that there could be no question that the malignant process was responsible for the death of the animal. In the other, designated as "accidental," the distribution of tumor was usually more limited, and except that a site such as the spine or jaws was involved, it has been assumed that death would not have occurred at this time. It is obvious that in an estimation of degrees of malignancy based upon comparative mortality rates, the numbers of accidental fatalities possess far less significance than those in the category of malignant deaths.

A considerable number of rabbits survived the observation period of 2 months. In some of them, however, the distribution of metastases was such that it is probable that death would eventually have occurred as a result of the tumor process. Growths in both suprarenal glands or in the facial and jaw bones are instances of this type of disease. These cases have been classified as "probable deaths." On the other hand, there were instances of surviving animals in which a few foci of tumor were found, but upon the basis of the distribution and state of these growths, they have been classified as "probable recoveries."

The number of foci of metastases refers to the number of organs or tissues involved, not to the actual numbers of secondary growths, and consequently, the expressions "foci of metastases," "distribution of metastases" or "metastatic rate" are used rather than "number of metastases."

TABLE I.

Virus-Free Tumor in Normal and Immune Rabbits.

Experiment	Group	Time after Virus III injection	No. of rabbits	Primary tumors	Mortality						Metastases					No. of metastatic foci		Probable deaths	Actual and probable recoveries	
					Total		Malignant cases		Accidental deaths		Incidence		Total foci	Relative rate	Actual rate	Deaths	Survivors		No.	Per cent
					No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent								
		days																		
I	N		9	All +	3	33.3	2	22.2	1	11.1	6	66.7	62	6.9	10.3	14, 20, 22	3, 2,	0 in 5	1	55.5
	I	39	9	All +	1	11.1	1	11.1	0	—	3	33.3	23	2.6	7.7	19	2, 2	0 in 6	1	77.7
II	N		10	All +	1	10.0	1	10.0	0	—	6	60.0	41	4.1	6.8	25	4, 4, 3, 3, 2	0 in 4	1	80.0
	I	25	9	8 +	1	11.1	0	—	1	11.1	5	55.5	21	2.3	4.2	11	7, 1, 1, 1	0 in 4	1	77.7
III	N		10	All +	1	10.0	1	10.0	0	—	4	40.0	32	3.2	8.0	21	8, 2, 1	0 in 6	1	80.0
	I	28	11	All +	0	—	0	—	0	—	6	55.5	20	1.8	3.3	—	7, 6, 3, 2, 1, 1	0 in 5	0	100.0
Total	N		29		5	17.2	4	13.8	1	3.4	16	55.2	135	4.7	8.4	102	32	0 in 15	4	68.9
	I		29		2	6.9	1	3.4	1	3.4	14	48.3	64	2.2	4.6	30	34	0 in 15	3	82.7

N = normal rabbits; I = rabbits immune to Virus III.

TABLE II.

Virus-Bearing Tumor in Normal and Immune Rabbits.

Experiment	Group	Time after Virus III injection	No. of rabbits	Primary tumors	Mortality						Metastases					No. of metastatic foci		Probable deaths	Actual and probable recoveries	
					Total		Malignant cases		Accidental deaths		Incidence		Total foci	Relative rate	Actual rate	Deaths	Survivors		No.	Per cent
					No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent								
		days																		
IV	N		10	All +	4	40.0	4	40.0	0	—	6	60.0	101	10.1	16.8	30, 32, 16, 12	9, 2	0 in 4	5	50.0
	I	28	10	All +	2	20.0	1	10.0	1	10.0	6	60.0	40	4.0	6.7	25, 6	4, 2, 2, 1	0 in 4	8	80.0

N = normal rabbits; I = rabbits immune to Virus III.

TABLE III.

Virus-Bearing Tumor in Normal Rabbits. Virus-Free Tumor in Immune Rabbits.

Experiment	Group	Time after Virus III injection	No. of rabbits	Primary tumors	Mortality						Metastases					No. of metastatic foci		Probable deaths	Actual and probable recoveries	
					Total		Malignant cases		Accidental deaths		Incidence		Total foci	Relative rate	Actual rate	Deaths	Survivors		No.	Per cent
		days			No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent								
V	N		10	All +	5	50.0	4	40.0	1	10.0	7	70.0	74	7.4	10.6	21, 17, 16, 9, 7	3, 1	0 in 3	0	5 50.0
	I	39	9	All +	1	11.1	1	11.1	0	—	3	33.3	23	2.6	2.6	19	2, 2	0 in 6	1	7 77.7
VI	N		10	All +	5	50.0	3	30.0	2	20.0	7	70.0	75	7.5	10.7	21, 15, 14, 14, 6	3, 2	0 in 3	0	5 50.0
	I	20	10	9 +	1	10.0	0	—	1	10.0	1	10.0	10*	1.0	10.0	10		0 in 9	0	9 90.0
VII	N		11	All +	4	36.4	3	27.3	1	9.1	9	81.9	74	6.7	8.2	26, 19, 14, 6†	4, 2, 1, 1, 1	0 in 2	0	7 63.6
	I	25	9	8 +	1	11.1	0	—	1	11.1	5	55.5	21	2.3	4.2	7	11, 1, 1, 1	0 in 4	1	7 77.7
VIII	N		10	All +	4	40.0	4	40.0	0	—	6	60.0	101	10.1	16.8	32, 30, 16, 12	9, 2	0 in 4	1	5 50.0
	I	28	11	All +	0	—	0	—	0	—	6	55.5	20	1.8	3.3	—	7, 6, 3, 2, 1, 1	0 in 5	0	11 100.0
Total	N		41		18	43.9	14	34.1	4	9.8	29	70.4	324	7.9	11.0	70	29	0 in 12	1	22 53.7
	I		39		3	7.7	1	2.6	2	5.1	15	38.5	74	1.9	4.9	36	37	0 in 24	3	33 84.6

N = normal rabbits; I = rabbits immune to Virus III.

*Number of metastatic foci estimated. † Complicating empyema.

TABLE IV.

Virus-Free Tumor in Normal Rabbits. Virus-Bearing Tumor in Immune Rabbits.

Experiment	Group	Time after Virus III injection	No. of rabbits	Primary tumor	Mortality						Metastases					No. of metastatic foci		Probable deaths	Actual and probable recoveries	
					Total		Malignant cases		Accidental deaths		Incidence		Total foci	Relative rate	Actual rate	Deaths	Survivors		No.	Per cent
					No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent								
		days																		
IX	N		10	All +	1	10.0	1	10.0	0	—	4	40.0	32	3.2	6.7	21	8, 2, 1 0 in 6	0	9	90.0
	I	28	10	All +	2	20.0	1	10.0	1	10.0	6	60.0	40	4.0	8.0	25, 6	4, 2, 2, 1 0 in 4	0	8	80.0

N = normal rabbits; I = rabbits immune to Virus III.

The distribution of secondary growths has been considered upon both a relative and an actual basis, the former including all animals of a group, while the latter takes into account only those rabbits in which metastases were found. The actual rate is obviously accentuated by individual rabbits with large numbers of foci, so that from a group standpoint the relative rate is a fairer index of comparative metastatic involvement. For other purposes, such as a numerical comparison of the uniformity of tumor distribution, both rates are of value.

Results.

The results of 9 experiments consisting of both clinical and post-mortem observations are summarized in Tables I, II, III and IV.

Table I contains data obtained in 3 experiments in which the virus-free tumor was inoculated in normal rabbits and in rabbits injected 25, 28 or 39 days previously with Virus III. Table II consists of the data of one experiment in which the virus-bearing tumor was used in an immunized group of rabbits; Virus III had been injected 28 days before the tumor. In the next 4 experiments, Table III, the pathological picture in normal rabbits induced by the virus-bearing tumor is contrasted with that of the virus-free tumor in rabbits injected 20, 25, 28 or 39 days previously with Virus III. Finally, Table IV summarizes the results of a single experiment in which the behavior of the virus-free tumor in normal rabbits was compared with that of the virus-bearing tumor in rabbits injected 28 days previously with Virus III.

DISCUSSION.

Before discussing the results of the experiments in which the course and character of the malignant disease in rabbits immune to Virus III was investigated, certain features of this study which must be considered in interpreting and evaluating the results should be briefly mentioned.

Because of the variability in the manifestation of the disease induced by this tumor, and in particular, the seasonal character of these variations (5), it is important in an investigation of comparisons of the disease under diverse conditions to carry out experiments at different seasons of the year. This has been done in the present instance as shown by the following dates of tumor inoculation:

Experiments I and V.....March 13 and 18, 1925.
Experiment VI.....May 12 and 15, 1925.
Experiments II and VII.....October 27 and 28, 1925.
Experiments III, IV, VIII and IX.....November 24 and 25, 1925.

The state of different materials used for inoculation must be considered in comparing results of 2 series, one of which was inoculated with the tumor bearing the virus and the other with the virus-free tumor (Tables III and IV). Both strains were transferred at monthly intervals to groups of not less than 10 rabbits and although actively growing primary tumors were used for this purpose, there was no criterion which would enable one to say that the 2 tumors were alike in actual or potential qualities of growth. But the chance of using less favorable material was the same in one case as in the other, except for the fact, which will be discussed later, that the disease in rabbits immune to Virus III was less malignant than in normal animals, and in certain experiments the virus-free tumor used for inoculation was derived from the primary growth of this strain in an immune rabbit. The possibility that this condition of host immunity modified the growth capacity and malignant potentialities of the tumor cannot be disregarded, but on the other hand, it should be pointed out that there is no reason for assuming that such modifications were of the nature of fixed characteristics.

One must also consider the interval between the inoculation of the normal and of the immune groups of an experiment. This factor does not enter into the first 4 experiments in which both groups were inoculated on the same day or in the last 3 in which inoculations were carried out on succeeding days. But in the 5th experiment there were 5 days, and in the 6th experiment, there were 3 days intervening between the inoculation of the 2 groups. What effect, if any, such a time difference might have upon the course and character of the malignant disease cannot be predicted, and one might be disposed to ignore it because of its shortness as compared with the 2 months' duration of the experiments and attribute any marked divergence of the pathological picture in the 2 groups to other factors. We have repeatedly observed, however, definite variations in the plane or level of malignancy in series of rabbits inoculated at intervals of 2 weeks, and in some instances of 1 week, when the material used for inoculation was

apparently as favorable in one case as in the other and other conditions under experimental control were common to both sets of animals. On this account, a number of experiments in which the interval between the inoculation of the 2 groups was 1 week or longer have not been included in the present paper. It may be said, however, that the observations derived from these additional experiments are in general agreement with those reported.

The analysis of experimental data may conveniently begin with the first result of inoculation, namely, the primary tumor. It has been our experience with the intratesticular route of injection that a primary tumor rarely fails to develop. In the present series of normal rabbits no failures were observed, but in 2 of the immune animals no tumor appeared. Although this proportion of failures is small, and may be entirely due to an error in the technic of inoculation, it is of interest that they occurred in the immune and not in the normal series. No attempt was made to measure the rate of growth and ultimate size of the primary tumors nor the speed and extent of regression in the instances in which this change occurred, but the general impression obtained of the initial reaction was that the tumors tended to develop more slowly and more irregularly in immune than in normal rabbits.

It will be seen by reference to Table I that in 3 experiments the disease was considerably less severe in rabbits immunized to Virus III than in normal animals; the virus-free strain of the tumor was used in these experiments. The lower level of malignancy in the immune group of each experiment is clearly brought out by the lower mortality rate, the fewer instances of pronounced malignancy, the smaller number of metastatic foci, the lower relative and actual rates of these growths and the higher incidence of actual and probable recoveries. As far as incidence of metastases is concerned, there is no consistent difference in the 3 experiments, but if the data are combined, the incidence is slightly lower among the immune than among the normal rabbits, that is, 48.3 per cent as contrasted with 55.2 per cent.

One experiment is available in which the behavior of the virus-bearing tumor was studied in 10 normal rabbits and in 10 rabbits immunized to Virus III (Table II). Again, the disease was much milder in the immunized group. The mortality rate was only half as

great, and the incidence of well marked cases of malignancy was one-fourth as high as in the group composed of normal animals. There was a much lower number of metastatic foci with a consequent reduction in the relative rate of these growths and although there was no difference in the incidence of metastases, the actual rate, in which only the animals with metastases are considered, was much smaller in the immune group, that is, 6.7 as compared with 16.8 in the normals.

The next comparison has been made with the virus-bearing tumor in normal and the virus-free tumor in immunized rabbits (Table III). The results of the 4 experiments are in general conformity with the others, but the contrast between the level of malignancy displayed by the normal groups and the very mild character of the disease of the immune series is even more pronounced. In each experiment, for instance, there were 3 or 4 cases of outspoken malignancy among the normal groups, but there was only 1 such case among all the immunes of the 4 experiments. There was also a much lower incidence of metastases in 3 immune groups, while in the 4th (Experiment VIII) it was slightly lower. As far as the numbers of metastatic foci and the relative and actual rates of these growths are concerned, the values for the immune groups are uniformly smaller than those for the normals.

In the last experiment, observations of immune rabbits inoculated with virus-bearing tumor are compared with those of normal rabbits inoculated with virus-free tumor (Table IV). The results of this experiment are not in accord with the others. Thus, there was the same incidence of pronounced cases of malignancy and practically the same number of metastatic foci with comparable relative rates of secondary growths in both immune and normal groups. In 2 particulars, however, the disease of the immune group was more severe than that of the normals, namely, in the higher incidence of metastases and in the slightly smaller number of actual and probable recoveries. But the disease of the immune group was considerably less severe than that of a group of normal rabbits inoculated with the same material (Experiment IV, Table II), so that as far as the reaction of the host to this particular inoculum was concerned, the resistance of rabbits immune to Virus III was more effective than that of normal

animals. It is probable, therefore, that the discordant results of the experiment were associated with the other group of animals, namely, the normal rabbits inoculated with virus-free tumor. The disease which developed in these animals was very mild, but not as mild as in a group of immune rabbits inoculated with the same material (Experiment III, Table I). It is likely, therefore, that the particular results obtained in Experiment IX were largely influenced by the character of the virus-free material used for inoculation. In the earlier work with the virus-free tumor the strain was carried in normal rabbits, but later, Virus III immune animals were used because it was feared that a reinfection of the tumor might occur. During the period of these experiments, the same method of monthly transfer of the virus-free strain in immune rabbits and of the virus-bearing stock tumor in normal rabbits was employed, but the fact that the disease in immune animals was comparatively mild suggests that material from such sources might not be as favorable as transplants taken from normal rabbits. That is to say, from the standpoint of the animal factor, conditions tended to favor growth and development of the tumor in the case of the virus-bearing strain, while the reverse obtained with the virus-free strain. On the other hand, it is important to note that the virus-free strain after a sojourn in immunized animals was still capable of inducing a process of well marked malignancy, for there were 2 such occurrences in the normal rabbits of Experiments II and III (Table I). It would appear, therefore, that the comparable results obtained with the immune and normal groups of Experiment IX were due, not to the failure of the immune state as such to be associated with a comparatively mild disease, but to the low level of the malignant process which developed in the normal rabbits.

Finally, as a conclusion to the comparison of individual experiments, the data of 7 experiments (I, II, III, IV, VI, VIII and IX) have been combined. The other 2 experiments cannot be used for this purpose because the data of their immune, although not of their normal groups, appear in other experiments. The observations are derived from 69 normal and 70 immune rabbits and have been analyzed in the same manner as in individual experiments with the following results:

	Total deaths	Malignant cases	Metastases			
			Incidence	No. of foci	Relative rate	Actual rate
	per cent	*per cent*	*per cent*		*per cent*	*per cent*
Normals	27.53	23.19	56.50	444	6.43	11.37
Immunes	10.00	4.29	47.15	174	2.48	5.27

The result of this analysis brings out in a striking manner the fact that the character of the malignant process in rabbits immunized to Virus III was much less severe than in normal rabbits, and further, that this lowered plane of malignancy occurred despite relatively little difference in the incidence of metastases. Virus III immunity did not diminish the number of rabbits in which metastases were found, but the unfavorable effect of this state upon the disease was evidently exerted upon the development of certain of these secondary growths as shown by the total number of foci, together with their relative and actual rates. But this unfavorable effect did not invariably occur, for there were 2 instances of pronounced malignancy in 2 immune groups (Experiments I and IV), a ratio of, roughly, 1 in 10. It is evident, therefore, that the reaction of the exceptional animal is little, if at all, influenced by the presence of an immunity to Virus III, and the inclusion of these rabbits disturbs the tendency toward numerical uniformity of pathological manifestations otherwise obtaining in animal groups in which the tumor process is of low malignancy.

The question of the comparative effectiveness of the immune state of rabbits to Virus III with respect to its duration cannot be properly discussed at this time because of insufficient material. Rabbits injected with Virus III become refractory to subsequent injections of the virus (intradermal) within 6 to 8 days, and the sera of such animals show well marked virucidal properties within a fortnight. As far as is known, these conditions continue for at least 6 months. The present experiments were performed 20 to 39 days after the injection of Virus III when a high state of immunity to the virus was present, but it is impossible to say whether variations in the degree of immunity associated with different periods of duration, if such variations exist, could be satisfactorily demonstrated by means of the malignant disease.

Finally, attention should be drawn to the fact that the immunity to Virus III which follows the injection of the virus-bearing tumor does not appear to be associated with an unfavorable influence upon the malignant process. The time necessary for the development of an immunity under these circumstances may account for this result. In the case of rabbits inoculated with testicular tissue emulsions rich in virus content, the immune state is fully developed within 6 to 8 days, but this period is lengthened to 2 to 3 weeks after the injection of the virus-bearing tumor. This difference in time is presumably due to differences in the amount or state of the virus. Under circumstances of rapid testicular passage at 4 or 5 day intervals, the amount of active virus must be very large, while it is undoubtedly smaller in the case of the tumor transferred at monthly intervals as shown by the fact that although testicular inoculations of stock tumors 4 to 8 weeks old lead to an immunity to Virus III, no visible virus reaction is obtained by means of intracutaneous inoculation of the same material.

Since the findings of the experiments reported in this paper show that the tumor process was not as mild in normal rabbits inoculated with virus-bearing tumor as in comparable groups of rabbits in which a Virus III immune state was present at the time of inoculation, it would appear that the character of the tumor process as a whole was largely determined by conditions or factors obtaining during the first 2 weeks after inoculation. As far as this particular tumor is concerned, however, such an assumption is not entirely warranted. For instance, young primary tumors which have grown slowly and to a limited extent for the first 2 or 3 weeks may suddenly assume an active and rapid growth. It is reasonable to presume that a similar change takes place in any metastatic growths with the result that what was apparently a more or less controlled tumor process became an uncontrolled one. The balance which exists between the ability of the host to control the malignant disease on the one hand, and the capacity of the tumor process toward the continued growth of primary and metastatic tumors on the other, is obviously influenced by a variety of factors. Nevertheless, it would appear from the present experiments that the growth capacities of the virus-bearing transplant were not affected by a slowly developing immunity to Virus III in the same manner as in

the case of an immunity present at the time of inoculation. On the other hand, the failure of the tumor process to be influenced by a delayed virus immunity may be explained upon the basis of the effect which the virus exerts on the animal host. This aspect of the question is discussed in the accompanying paper dealing with the effect of Virus III on the malignant disease (4).

The effect of host immunity to Virus III upon the manifestations of this malignant tumor must be of an entirely non-specific nature. Virus III has been extensively studied in a large number of rabbits for 4 years and there is no indication that it produces tumors of any type. Although the stock tumor with which we have worked was found to be infected with Virus III, there is no reason for assuming that its presence was anything but a fortuitous occurrence and due to 2 factors, first, that this virus is indigenous to rabbits and second, that the tumor presents unusually favorable conditions for the growth and survival of certain viruses. Furthermore, a virus-free strain of the tumor has been found to possess the essential characteristics of malignancy exhibited by the virus-bearing strain, for it can be transplanted from rabbit to rabbit, it gives rise to metastatic growths and it has caused death. The effect of Virus III immunity upon the course and character of the neoplasm has, moreover, been observed in the disease induced by both virus-free and virus-bearing tumors. And it may be mentioned in this connection that similar effects have been observed in connection with concomitant infections which have affected the course of the tumor and also experimental infection with *Treponema pallidum*.*

The mechanism by which this effect is produced is not known. If one considers resistance or susceptibility to disease as a functional activity of the animal organism, then it is evident that the low plane of malignancy displayed by rabbits immunized to Virus III was brought about by factors which affected animal economy resulting in an increased or a more effective resistance to the tumor process.

SUMMARY AND CONCLUSIONS.

Experiments are reported in which were studied the course and character of a transplantable malignant neoplasm in normal rabbits and in rabbits immunized with a filterable virus, Virus III.

* Unpublished experiments of L. Pearce.

The disease which developed in immunized rabbits was extremely mild and much less severe than in normal animals.

The effect upon the tumor process displayed by Virus III immune rabbits in the direction of diminished malignancy is considered to be entirely non-specific in character, and the suggestion is made that it is accomplished through a more effective resistance of the host.

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EFFECT OF A FILTERABLE VIRUS (VIRUS III) ON THE GROWTH AND MALIGNANCY OF A TRANSPLANTABLE NEOPLASM OF THE RABBIT.

By LOUISE PEARCE, M.D., and THOMAS M. RIVERS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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In connection with the study of the effect of an immunity to Virus III on the course of a transplantable neoplasm of the rabbit (1), other problems relating to the tumor were investigated. The finding that the stock tumor (2) was infected with Virus III (3) which was being regularly transferred with it, suggested the question whether the presence of Virus III affected the growth and malignancy of the tumor. The fact that we were able to obtain the tumor without the virus and to transfer it from rabbit to rabbit furnished the means for an experimental study of this problem, the results of which are reported in the present paper.

EXPERIMENTAL.

Materials and Method.

The general plan of the experiments consisted of the study of parallel series of rabbits inoculated with the virus-bearing and the virus-free strains of the tumor. The dates of inoculation were as follows:

Experiment I	January 14 and 17, 1925.
Experiment II	February 16, 1925.
Experiment III	March 13 and 18, 1925.
Experiment IV	April 15 and 17, 1925.
Experiment V	October 27 and 28, 1925.
Experiment VI	November 24 and 25, 1925.
Experiment VII	November 24 and 25, 1925.

Neoplasm.—The tumor which is described fully in earlier papers (2) is considered to be of epithelial origin.

Virus III.—This has been described in the preceding paper (1) and more detailed accounts have previously been published (3).

TABLE I.

Comparison of Virus-Bearing and Virus-Free Tumor in Normal Rabbits.

Experiment	Group	No. of rabbits	Primary tumors	Mortality						Metastases					No. of metastatic foci			Probable deaths	Actual and probable recoveries	
				Total		Malignant cases		Accidental deaths		Incidence		Total foci	Relative rate	Actual rate	Deaths	Survivors			No.	Per cent
				No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent									
I	V +	10	All +	2	20.0	0	—	2	20.0	4	40.0	26	2.6	6.5	10, 7	5, 4	0 in 6	1	7	70.0
	V −	9	All +	6	66.6	2	22.2	4	44.4	9	100.0	61	6.8	6.8	15,* 11, 9, 7, 5, 3	8, 2, 1	0 in 0	1	2	22.2
II	V +	9	All +	4	44.4	4	44.4	0	—	5	55.5	81	9.0	16.2	30, 17, 15, 15	4	0 in 4	0	5	55.5
	V −	9	All +	6	66.6	2	22.2	4	44.4	6	66.6	70	7.7	11.7	22, 15, 10, 9, 9, 5	—	0 in 3	0	3	33.3
III	V +	10	All +	5	50.0	4	40.0	1	10.0	7	70.0	74	7.4	10.6	21, 17, 16, 9, 7	3, 1	0 in 3	0	5	50.0
	V −	9	All +	3	33.3	2	22.2	1	11.1	6	66.6	62	6.9	10.3	22,* 20, 14	3, 2, 1	0 in 3	1	5	55.5
IV	V +	10	All +	2	20.0	0	—	2	20.0	4	40.0	26	2.6	6.5	12, 8	4, 2	0 in 6	2	6	60.0
	V −	10	9 +	3	30.0	2	20.0	1	10.0	8	80.0	63	6.3	7.9	19,* 14, 12	7, 5, 4, 1, 1	0 in 2	2	5	50.0
V	V +	11	All +	4	36.4	3	27.3	1	9.1	9	81.9	74	6.7	8.2	26, 19, 14, 6†	4, 2, 1, 1, 1	0 in 2	0	7	63.6
	V −	10	9 +	1	10.0	1	10.0	0	—	6	60.0	41	4.1	6.8	25	4, 4, 3, 3, 2	0 in 4	1	8	80.0
VI	V +	10	All +	4	40.0	4	40.0	0	—	6	60.0	101	10.1	16.8	32, 30, 16, 12	9, 2	0 in 4	1	5	50.0
	V −	10	All +	1	10.0	1	10.0	0	—	4	40.0	32	3.2	8.0	21	8, 2, 1	0 in 6	1	8	80.0
Total	V +	60	All +	21	35.0	15	25.0	6	10.0	35	58.3	382	6.48	10.92				4	35	58.3
	V −	57	55 +	20	35.1	10	17.6	10	17.6	39	68.4	329	5.78	8.44				6	31	54.4

TABLE I.—*Concluded.*

Comparison of Virus-Bearing and Virus-Free Tumor in Virus III Immune Rabbits.

Experiment	Group	No. of rabbits	Primary tumors	Mortality						Metastases					No. of metastatic foci		Probable deaths	Actual and probable recoveries	
				Total		Malignant cases		Accidental deaths		Incidence		Total foci	Relative rate	Actual rate	Deaths	Survivors		No.	Per cent
				No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent								
VII	V +	10	All +	2	20.0	1	10.0	1	10.0	6	60.0	40	4.0	6.7	25, 6	4, 2, 2, 1 0 in 4	0	8	80.0
	V −	11	All +	0	—	0	—	0	—	6	55.5	20	1.8	3.3	—	7, 6, 3, 2, 1, 1 0 in 5	0	11	100.0
Total of all experiments	V +	70	All +	23	32.9	16	22.9	7	10.0	41	55.9	422	6.2	10.3			4	43	61.4
	V −	68	66 +	20	29.4	10	14.7	10	14.7	45	66.2	349	5.1	7.8			6	42	61.8

V+ = virus-bearing tumor; V− = virus-free tumor.

* Number of metatastic foci estimated.

† Complicating empyema.

TABLE II.

Analysis of Metastatic Foci in All Fatal Cases.

Group	4th wk.	5th wk.	6th wk.	7th wk.	8th wk.
Virus-bearing tumor	II 30* II 17* II 15* IV 12 IV 8 V 26* VI 30*	II 15* III 21* III 16* V 19* VI 32* VI 16* VII 6	V 6 VII 25*	I 7 III 17* III 9* III 7 V 14* VI 12*	I 10
Virus-free tumor	I 5 II 9 II 5 III 14 VI 21*	I 3 II 22* II 10 III 22*† III 20* V 25*	I 7 I 15*† II 15* IV 12	I 9 II 9 IV 19*†	I 1†* 14*

Deaths 4th and 5th wks.	Malignant cases		Total foci	Foci in malignant cases	
	No.	Per cent		No.	Per cent
V + 14	11	78.6	263	237	90.1
V − 11	5	45.5	156	110	70.5

Deaths 6th, 7th and 8th wks.	Malignant cases		Total foci	Foci in malignant cases	
	No.	Per cent		No.	Per cent
V + 9	5	55.5	107	77	72.0
V − 9	5	55.5	111	74	66.8

Total deaths	Malignant cases		Total foci	Foci in malignant cases	
	No.	Per cent		No.	Per cent
V + 23	16	69.6	370	314	84.9
V − 20	10	50.0	267	173	64.8

Roman numerals refer to number of experiment; Arabic to number of metastatic foci.

* Instances of well marked malignancy.

† Number of foci estimated.

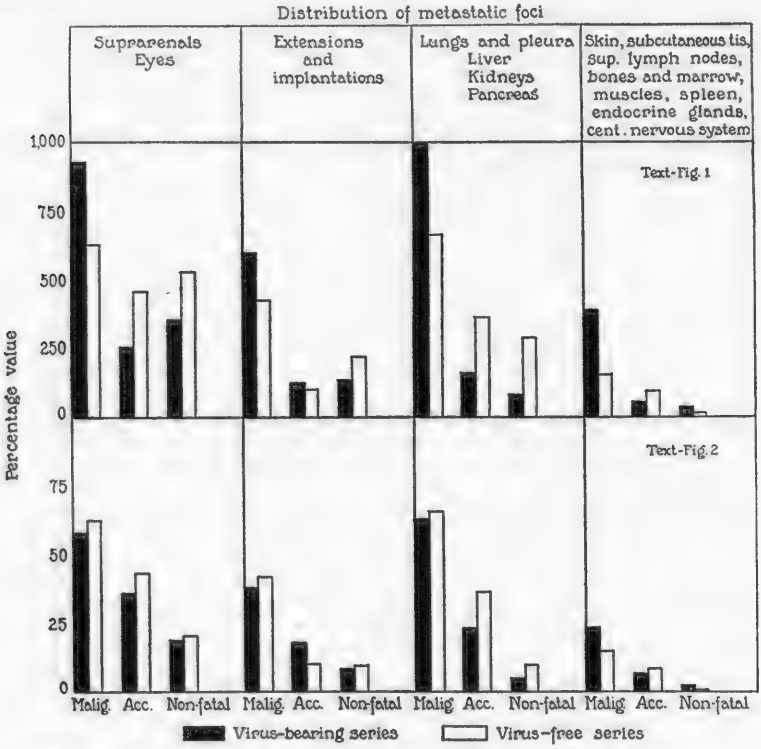
TABLE III.

Distribution of Metastatic Foci.

Group	No. of rabbits	I. Suprarenals and eyes				II. Extensions and implantations				III. Lungs				IV. Skin, subcut. tissue, super. lymph nodes, etc.				Total foci	
		No. of foci	Per cent	Foci per rabbit	Per cent	No. of foci	Per cent	Foci per rabbit	Per cent	No. of foci	Per cent	Foci per rabbit	Per cent	No. of foci	Per cent	Foci per rabbit	Per cent	No.	Per rabbit
Malignant cases																			
V +	16	37	925.0	2.31	57.8	112	593.6	7.0	37.1	50	1000.0	3.12	62.5	115	379.5	7.2	23.7	314	19.6
V −	10	25	625.0	2.5	62.5	79	418.7	7.9	41.9	33	660.0	3.3	66.0	47	155.1	4.7	15.5	184	18.4
Accidental deaths																			
V +	7	10	250.0	1.43	35.7	23	121.9	3.3	17.4	8	160.0	1.42	22.9	15	50.0	2.1	7.1	56	8.0
V −	10	17	425.0	1.7	42.5	20	106.0	2.0	10.6	18	360.0	1.8	36.0	28	92.4	2.8	9.2	83	8.3
Non-fatal cases																			
V +	18	14	350.0	0.78	19.4	25	132.5	1.4	7.4	4	80.0	0.2	4.4	9	29.7	0.5	1.7	52	2.9
V −	25	21	525.0	0.84	21.0	41	217.3	1.6	8.7	14	280.0	0.6	11.2	6	19.8	0.2	0.8	82	3.3
Total																			
V +	41	61	1525.0	1.48	37.2	160	848.0	3.9	20.7	62	1240.0	1.5	30.2	139	458.7	3.4	11.2	422	10.3
V −	45	63	1575.0	1.4	35.0	140	742.0	3.1	16.5	65	1300.0	1.4	28.9	81	267.3	1.8	5.9	349	7.8

Virus-Free Strain of the Neoplasm.—This has been described in an earlier report (4) and in the preceding paper (1).

The experiments reported in this paper were carried out in the same manner as those previously reported (1).

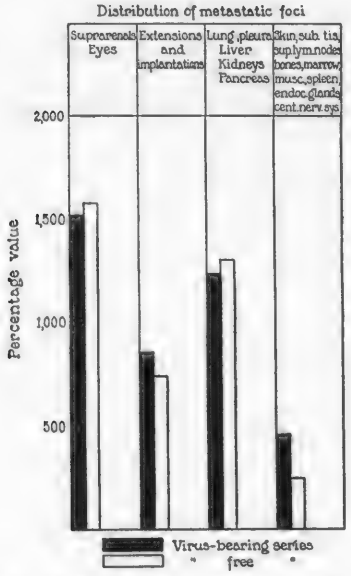


TEXT-FIG. 1. Distribution of metastatic foci in different types of disease.

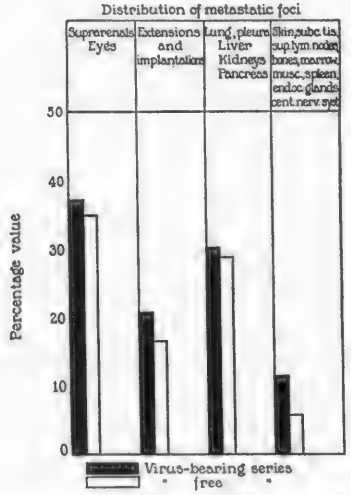
TEXT-FIG. 2. Average distribution per animal of metastatic foci in different types of disease.

Method of Analysis of Results.—Clinical and postmortem observations have been analyzed in the same way as those given in the preceding paper (1). The secondary growths have also been considered in relation to the organs and tissues involved.

The percentage estimations of metastatic distribution have been based upon the number of foci theoretically possible as shown by the actual location of metas-



TEXT-FIG. 3. Distribution of metastatic foci in all cases.



TEXT-FIG. 4. Average distribution per animal of metastatic foci in all cases.

tases in the first 20 generations of tumor animals (5). Certain obvious objections may be made to this method. Such organs as the liver or kidneys, for example, may show numerous tumors or only a few which destroy little of the organ. But, the general character of the disease, whether of high, moderate or low malignancy, is shown by grouping the metastatic foci in the following divisions:

I. Suprarenals and eyes	4	possible foci
II. Extensions and implantations to the retroperitoneal and mediastinal tissues, omentum, mesentery and parietal peritoneum	19	" "
III. Lungs and pleura, liver, kidneys and pancreas	5	" "
IV. Skin and subcutaneous tissue, superficial lymph nodes, muscles, heart and pericardium, bones and bone marrow, glands of internal secretion with the exception of the suprarenals, the spleen and the central nervous system	30	" "

This arrangement which has been employed in analyzing the results of other tumor experiments (6) was selected upon the basis of observations of several hundred rabbits inoculated with this tumor. Suffice it to say that in those animals in which the most malignant disease develops and in which death occurs within 3 to 5 weeks after inoculation, there is usually a widespread distribution of secondary growths to many organs and tissues, including the skin, muscles, bones and bone marrow and the endocrine glands which are practically never involved in cases of low malignancy. In those animals in which the disease is very mild, metastases may be found only in the eyes or suprarenal glands or in the facial and jaw bones and cranial sinuses which also, of course, are frequently involved in instances of a severe disease. All gradations are found between these extremes of malignancy.

Results.

The results of the 7 experiments here reported consist of the clinical and postmortem observations of 138 rabbits, 70 of which were inoculated with the virus-bearing and 68 with the virus-free strain of the tumor. For convenience of discussion, these observations are summarized in Table I.

An analysis of the metastatic foci found in all fatal cases is based upon the time at which such deaths occurred with reference to the time of inoculation (Table II).

A further analysis of the character of the disease takes into account the various organs and tissues actually involved by metastases (Table III). The charts of Text-figs. 1 to 4 are graphic representations of the numerical values of Table III.

DISCUSSION.

The experiments reported were undertaken for the purpose of ascertaining whether the presence of Virus III in a transplantable neoplasm of the rabbit influenced the character of the malignant disease. Because of the variability of the tumor process which may be influenced by many factors, the value that can be attached to differences in disease manifestations must be interpreted with due consideration of the circumstances of the experiment. In the present study the factors which could be controlled were common to both animal groups of each experiment.

The most important variable which could not be controlled was the inoculating material itself which, of course, was derived from different sources and there was no means of ascertaining whether there were actual or potential differences of growth capacity in the 2 tissues. It might be said that this difference in material constitutes an objection to the comparison of results, but since the experiments necessitated the presence of the virus in one strain of the tumor and its absence in the other, the use of transplants from different sources was unavoidable. In both strains the tumor employed for transfer was carefully selected with the intention of providing as favorable material in the one case as in the other. With the virus-bearing strain (the stock tumor) transfers were regularly made at monthly intervals as had been our custom for several years. This method was also followed with the virus-free strain during the time of these experiments, but immediately preceding the first experiment, transfers had been made more frequently which may have influenced the results obtained. The material used in the last 3 experiments was obtained from rabbits which had been immunized to Virus III, and as has been pointed out in the preceding paper (1), there is some evidence to show that material from such a source is not as favorable as that obtained from normal rabbits. This feature will subsequently be discussed more fully, but it may be stated now that material subjected to such supposedly deleterious influences as freezing, thawing and grinding in the frozen state before being used for inoculation appeared to have no appreciable effect on the character of the disease produced (7). While these procedures were extraneous as contrasted with possible effects of the virus

within the tumor, both being subjected to the influence of the animal organism, the results of the experiments referred to show that chance inequalities of material used for inoculation may not be a factor of any considerable importance where extreme variations of malignancy are concerned.

The first impression obtained from the summary of the clinical and postmortem observations in Table I is the variability of the disease picture in both the virus-bearing and the virus-free groups. This feature was, with 2 exceptions, more pronounced in the case of the virus-bearing strain, as shown by percentage variation values of various disease manifestations. In the case of the total mortality rate, the virus-free series was the more variable while the percentage variation of metastatic incidence was the same in both series.

Virus-bearing tumor						Virus-free tumor					
Total mortality	Malignant cases	Incidence of metastases	Foci of metastases	Relative rate	Actual rate	Total mortality	Malignant cases	Incidence of metastases	Foci of metastases	Relative rate	Actual rate
per cent	*per cent*	*per cent*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*
50.0	44.4	81.9	101	10.1	16.8	66.6	22.2	100.0	70	7.7	11.7
44.4	40.0	70.0	81	9.0	16.2	66.3	22.2	80.0	63	6.9	10.3
40.0	40.0	60.0	74	7.4	8.2	33.3	22.2	66.6	62	6.8	8.0
36.0	27.3	60.0	74	6.7	6.9	30.0	20.0	66.6	61	6.3	7.8
20.0	10.0	55.5	40	4.0	6.7	10.0	10.0	60.0	41	4.1	6.8
20.0	0	40.0	26	2.6	6.5	10.0	10.0	55.5	32	3.2	6.8
20.0	0	40.0	26	2.6	6.5	0	0	40.0	20	1.8	3.3
Percentage variation.....33.7	73.3	19.2	42.1	42 2	36.3	68.1	48.0	19.7	32.4	36.3	24.4

If the results of all experiments are combined, the disease produced by the virus-bearing strain appears to be more severe (Table I). The number of cases of well marked malignancy was distinctly greater, 22.9 as contrasted with 14.7 per cent; there were more foci of metastases, 422 as compared with 349, and in addition, the relative and actual metastatic rates were higher, 6.2 and 10.3 as contrasted with 5.1 and 7.8 respectively. The incidence of metastases, however, was slightly lower, 55.9 as contrasted with 66.2 per cent. There was no

difference in the proportions of actual and probable recoveries, 61.0 per cent of each series falling in this category and little difference in the small numbers of probable deaths, so that the observations of the fatal cases are of particular importance.

In the analysis which has been made of the fatal cases (Table II), special attention is given to the time of death since the majority of rabbits with a process of pronounced malignancy die within 3 to 5 weeks after inoculation. Reference to Table II shows that approximately the same proportion of deaths in each group occurred within the 4th and 5th weeks after inoculation, that is, in the case of the virus-bearing series 14 out of 23 cases or 60.9 per cent and 11 out of 20 or 55.0 per cent in the virus-free series. But there was a decided difference in the incidence of cases of well marked malignancy, for 11 of the 14 deaths (78.6 per cent) in the virus-bearing series were of this type as contrasted with 5 of the 11 deaths (45.5 per cent) in the virus-free group. The proportion of metastatic foci in these malignant cases to the total number of foci found in all fatal cases of the 4th and 5th weeks shows a striking difference, that is, virus-bearing, 90.1 per cent; virus-free, 70.5 per cent. When the deaths which occurred in the last 3 weeks of the observation period are similarly considered, however, there is little difference between the 2 groups.

If these comparisons be made with all deaths irrespective of time, the values obtained as given below would indicate that the more severe condition developed in the disease produced by the virus-bearing tumor.

Group	Total deaths	Malignant cases		Total metastatic foci	Foci in malignant cases	
		No.	Per cent		No.	Per cent
Virus-bearing	23	16	69.6	370	314	84.9
Virus-free	20	10	50.0	267	173	64.8

The higher incidence of cases of well marked malignancy in the virus-bearing series is of considerable significance in view of the total number of animals comprising each group (Table I). There was practically no difference in the total mortality rates of the 2 series, however, nor in the time distribution of all deaths, but there was a considerable

difference in the incidence and time of the so called accidental deaths as shown by the following data:

Group	No. of rabbits	Total deaths		Accidental deaths		Deaths 4th and 5th wks.		
						Total	Accidental	
		No.	Per cent	No.	Per cent		No.	Per cent
Virus-bearing	70	23	32.9	7	30.4	14	3	21.4
Virus-free	68	20	29.4	10	50.0	11	6	54.5

The accidental deaths are directly caused by the particular location of a metastasis such as the spine, and other secondary growths are not necessarily numerous or destructive, especially if death occurs within the first few weeks. These cases cannot be dismissed as entirely lacking in malignant potentialities, however, for it is not impossible that at least some of them would have subsequently died from a more extensive tumor process had the immediate cause of an early death not occurred. But, as far as the average numerical distribution of foci in the different types of fatal cases is concerned, the following figures show little difference between the virus-bearing and the virus-free series:

Deaths Due to Well Marked Malignancy.

Group	4th and 5th wks.			6th, 7th and 8th wks.		
	No. of rabbits	No. of metastatic foci	Rate	No. of rabbits	No. of metastatic foci	Rate
			per cent			*per cent*
Virus-bearing	11	237	21.5	5	77	15.4
Virus-free	5	110	22.0	5	74	14.8
Accidental Deaths.						
Virus-bearing	3	26	8.7	4	30	7.5
Virus-free	6	46	7.7	4	37	9.3

As far as the analysis of results has been carried, the chief points of difference between the 2 series of rabbits are (1) the higher incidence

of fatal cases with a tumor process severe enough to be classified as "malignant" in the virus-bearing series, and (2) a lower incidence of "malignant" cases but a higher incidence of early "accidental" deaths in the virus-free series.

The question at once arises as to how much importance should be attached to such points of difference as indicating variations of disease severity which could be ascribed, either directly or indirectly, to the presence or absence of Virus III in the tumor. Before this subject is discussed, the character of the disease should be considered from the standpoint of the organs and tissues involved. In such an analysis it is convenient to adopt some such plan as was outlined in the section on Materials and Method which consists in grouping the secondary tumors observed at postmortem examination into major divisions corresponding to the distribution of metastases which have been found in association with tumor processes of various degrees of severity (Table III).

The first column of each of the 4 divisions of Table III contains the total number of foci found while the figures in the second column represent the average number of foci per animal. The percentage values have been calculated upon the basis of the total number of theoretically possible growths in these sites. Text-figs. 1 to 4 are graphic representations of the values of Table III. The analysis includes all cases in which secondary growths were found and the classification of these cases previously employed has been followed, that is, malignant cases, accidental deaths and animals surviving the observation period.

Reference to Table III and to Text-fig. 1 shows that as far as the malignant cases are concerned, there were many more metastatic foci in all 4 divisions of secondary growths in the virus-bearing than in the virus-free series, which is to be expected in view of the larger number of these cases in this group. When the average value per animal is considered, however, there is little difference between the 2 series except in one important division (IV), namely, that which includes the skin, muscles, bones and endocrine glands (Text-fig. 2). Metastases to these tissues and organs rarely occur except in cases of well marked malignancy. It must be remembered, moreover, that the number of theoretically possible foci in this division is much larger than in the others, so that small differences are significant, as in the present in-

stance in which the values for the virus-bearing and virus-free series were 7.2 and 4.7 per rabbit, respectively. The distribution of secondary growths would indicate, therefore, that the tumor process in the cases of well marked malignancy was more severe in the virus-bearing than in the virus-free series.

In the group of accidental deaths, on the other hand, the disease was slightly but definitely more severe in the virus-free group (Table III; Text-figs. 1 and 2). The values for this group in the 1st, 3rd and 4th divisions of metastatic foci are all larger than those of the virus-bearing series. In the 2nd division, that of extensions and implantations, the figures for the virus-bearing series are the larger, but as has been pointed out, tumors in this category as well as those in the suprarenal glands and the eyes (Division I) possess far less significance from the standpoint of disease severity than do those in such locations as the lungs, liver, kidneys, skin, muscles, bones and other glands of internal secretion (Divisions III and IV). The reason for the higher incidence of accidental deaths in the virus-free series is not clear, but the fact that the disease in these cases was more severe in the virus-free than in the virus-bearing group suggests that under other circumstances, such as a larger number of animals or another experimental period, the malignant level of the entire virus-free series might more nearly approach that of the virus-bearing series.

If the figures for these disease types—the malignant cases, the accidental deaths and the non-fatal cases—are combined and analyzed in the same manner (Table III; Text-figs. 3 and 4), it is seen that there was practically no difference between the virus-bearing and the virus-free groups in regard to extensions and implantations (Division II), and to the distribution of metastases to the suprarenals and eyes (Division I), and to the lungs, liver and kidneys (Division III). On the other hand, the more frequent involvement of the skin, subcutaneous tissues, muscles, bones, heart, central nervous system and glands of internal secretion (Division IV) in the virus-bearing series points to a tumor process of a somewhat higher malignancy than that of the virus-free series. The conclusion from these analyses is in accord with those previously arrived at from a consideration of such features of the disease as the mortality rate, the type of fatal case and the number and rate of metastatic foci.

The combined observations of all experiments have so far been considered but if the results of individual experiments are compared, it is seen that they are not entirely constant (Table I). The degree of malignancy in Experiments II, III, V, VI and VII was greater in the virus-bearing groups as shown by the incidence of malignant cases, the total number of metastatic foci and the relative and actual rates of the growths. But in Experiments I and IV, the disease of the virus-free groups was more severe. In these 2 experiments the tumor process of the virus-bearing series was very mild, much more so, in fact, than in any other group in any experiment except the last, No. VII, in which the disease of both groups was benign. Rabbits immunized to Virus III were employed in this experiment, which condition is associated with a disease of low malignancy (1).*

Pronounced variations in malignancy in groups of rabbits inoculated with the stock tumor at consecutive monthly intervals have been observed over long periods. From an analysis of the first 20 generations of the tumor, it appeared that the principal factors concerned in determining the results of transplantation were adaptation to passage and variations in meteorological conditions that prevailed during the time the experiments were carried out,—the one affecting the energy of cell growth, and the other affecting animal economy (7). It must be assumed that meteorological influences would be exerted over both groups of animals in each of the present experiments. On the other hand, the possibility that such an agent as Virus III might affect the energy of cell growth should be considered. The filterable viruses are intimately associated with cells, and frequently with young cells in particular, and it is possible that with the tumor some biological effect would be induced by the presence of Virus III, perhaps of the nature of a stimulation to cell growth. In the case of vaccine virus and Virus III, for example, the idea of cell stimulation, probably in connection with cell injury, is supported by the histological picture of early lesions of the cornea and skin of rabbits.

* In Experiment VII, the disease was not as mild in the virus-bearing as in the virus-free group. It is of interest to note that of the 20 metastatic foci in the virus-free group (Table I), 1 was healed and 13 were largely or wholly necrotic. In the virus-bearing group, on the other hand, a similar condition was noted in but 3 of the 40 metastatic foci.

The growth capacity of the tumor cells may be so great that the stimulation to cell growth induced by an agent like Virus III would be entirely negligible. On the other hand, if such an influence is especially exerted upon young cells, and if it results in an increase in the rate of cell multiplication, a more vigorous growth of the early primary tumor would probably take place, which might lead to the production of a more severe disease. However, the tumor process is affected by a variety of factors and conditions, some of which might favor and others oppose the hypothetical influence of Virus III and the mildness of the disease in Experiments I and IV may be examples of the successful opposition, from an unknown cause, of the more usual effect associated with the virus.

Attention should also be called to the fact that although the disease of the virus-free groups was more uniform than that of the virus-bearing groups with respect to certain pathological manifestations, there was a definite tendency toward a lower plane of malignancy in the last as compared with the first experiments. During the course of this study, both strains of the tumor were transferred at monthly intervals and as presumably favorable material was used in the one case as in the other. But immediately preceding the experiments, the virus-free strain had been transferred more frequently, and although no conclusive evidence is available that such a procedure promotes increased malignancy, other factors being equal, still the possibility should be considered. During the latter part of this work, the virus-free tumor was carried in rabbits which had been immunized to Virus III, and observations show that in them there is a definite tendency toward a disease of lowered malignancy (1). It is possible, therefore, that the inoculating material derived from Virus III immune animals was of a less favorable character and as such may have contributed to the decreased severity of the disease as it appeared in the virus-free groups of the last 3 experiments.

Among other factors which may have indirectly influenced the course of the process induced by the virus-bearing tumor, there is one in particular which merits special attention, namely, the effect of Virus III on the rabbit host. It will be recalled that a certain number of normal rabbits, 15.0 to 20.0 per cent on an average in this laboratory, have been found to be immune to Virus III. It has also been observed

that the tumor process tends to be less severe in rabbits which have been experimentally immunized to Virus III than in normal rabbits (1). From these facts, it would appear that the chance inclusion of one or more Virus III immune rabbits in a group of 10 animals would affect the experimental results in the direction of diminished malignancy. There was just as much chance, however, for immune rabbits to be included in the virus-bearing as in the virus-free groups. All rabbits inoculated with the virus-free tumor which were subsequently tested for an immunity to Virus III showed a typical cutaneous reaction, indicating the absence of an immunity. Preliminary virucidal tests of the sera of rabbits subsequently inoculated with the virus-bearing tumor were not carried out in the present series of experiments, so that it is impossible to say whether any Virus III immune animals were included in these groups.

On the other hand, one must take into account the possible effect of Virus III upon the course of the disease from the standpoint of the more immediate reaction of the animal host to the introduction of the virus with the tumor. At first glance, one might suppose that the severity of the tumor process would be diminished since the general plane of malignancy tends to be low in rabbits immunized to Virus III and rabbits inoculated with the virus-bearing tumor develop an immunity to the virus. It has been shown, however, that such is not the case, the probable explanation being that the immunity occasioned by the virus-bearing tumor develops more slowly than that intentionally caused by the injection of tissue emulsions rich in virus content (1). When an immunity is fully developed in connection with virus-bearing transplants, the tumor process has apparently reached the point when it is not affected by the immune state of the host, to the degree, at least, that is observed in rabbits which are already immune to Virus III at the time of tumor inoculation. On the other hand, the presence of the virus within the tumor affects the animal host since an immunity to it eventually develops, and it is not unlikely that during the development of this state, the response of the host to other pathogenic agents would be affected. Thus, in the present instance, the somewhat greater severity of the disease induced by the virus-bearing strain may be due to an increased susceptibility or a decreased resistance of the host as a collateral or indirect result of the

Virus III infection of the tumor transplant. In Experiments I and IV, in which the disease was unusually mild, it is not unreasonable to assume that there were present factors or conditions which operated to prevent this alteration in the reaction of the host to the tumor or which effectively opposed the result of such an alteration. An unusually low content or diminished activity of Virus III in the material used for inoculation or an already existing immunity to Virus III are factors which might operate in this manner.

The reciprocal effects of concomitant or superimposed disease conditions have received considerable attention, chiefly, however, from a clinical standpoint. That the subject is open to experimental investigation is shown by the results of the present study which suggest that the presence of one pathogenic agent, a filterable virus, is associated with certain variations in the disease induced by a second agent, the malignant tumor. That these variations are not of larger magnitude may be related to the virulence of the virus which is not high as ordinarily considered in terms of the usual criteria of animal reaction, so that a pronounced effect would hardly be expected.

In conclusion, it should be pointed out that the influence of an associated infection with Virus III as a factor concerned in determining variations in growth and malignancy of the tumor must be evaluated with due regard to the many other factors which influence the disease process. There is no indication, for instance, that extreme variations in malignancy could be accounted for on this basis, or that the orderly succession of periods of increasing and diminishing malignancy with a distinct and characteristic tendency to a seasonal distribution are affected by the presence or absence of Virus III in the tumor. The present observations, however, indicate that the presence of Virus III is usually associated with a higher degree of malignancy while the effect of an immunity to Virus III has been shown to be associated with a disease of diminished severity (1). These conditions probably account for some of the irregularities in results observed in individual animals and in certain groups of animals.

SUMMARY AND CONCLUSIONS.

A study of a malignant disease in rabbits has been made with reference to the presence or absence of a filterable virus, Virus III, in the

tumor. The results are analyzed from the standpoint of certain characteristic features of the tumor process in order to determine any differences in degrees of malignancy.

It was found that a more severe disease developed in the series in which the virus-bearing tumor was used than in the series in which the tumor was free of the virus, although the differences were not very marked and were not entirely constant.

The influence of Virus III as a factor affecting malignancy has been discussed from the standpoint of its possible effect upon (*a*) the tumor cells and (*b*) the host reaction. It has been suggested that the greater malignancy of the pathological process usually induced by the virus-bearing tumor is attributable to a change in the response of the host to the tumor, which change is of the nature of a decreased resistance associated with the reaction of the host to the virus infection.

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IMMUNOLOGICAL PROPERTIES OF A TYPICAL (S-PRODUCING) AND A DEGRADED (NON-S-PRODUCING) STRAIN OF TYPE II PNEUMOCOCCUS WITH SPECIAL REFERENCE TO PROTECTIVE ANTIBODIES.

By EMIDIO L. GASPARI,* WILLIAM L. FLEMING, and JAMES M. NEILL, Ph.D.

(*From the Department of Bacteriology and Immunology, Vanderbilt University Medical School, Nashville.*)

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INTRODUCTION.

The advance in the knowledge of the chemical nature of the carbohydrate substance (1–3) of Pneumococcus makes it desirable to obtain further evidence of its biological significance. Facts of interest in this connection have been furnished by the reports (4) of the changes in immunological properties exhibited by pneumococci which have lost the specialized cellular function of elaborating the soluble specific substance (carbohydrate). Reimann's (5) recent paper adds value to the earlier work (4), since his results indicate that the immunological differences accompanying the loss of the S-producing function can be explained upon the basis of Avery and Heidelberger's interpretation of the immunological relations of pneumococcus cell constituents.

It is the object of the present paper to present evidence of similar nature, but with special emphasis upon the immunological reactions involved in the protection of mice against virulent Type II pneumococci. The study consisted of a comparison of the immunological properties of two strains of pneumococci: a type-specific strain in which the S-producing function of the cell was highly developed; and a non-type-specific strain in which the S-producing function was completely lost. The comparison of the two strains from the stand-

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point of antipneumococcus protection of mice was investigated by active and passive immunity tests, and by the absorption of antipneumococcus serum with whole bacterial cells and with culture filtrates.

EXPERIMENTAL.

Strains of Pneumococci.—Two strains of pneumococci were employed in the investigation: (1) an S-producing[1] (Type II) strain; (2) a non-S-producing[1] strain derived by the "spontaneous degradation" of the first strain. *S-Producing Strain.*—Strain D_{39}, Type II (Hospital of The Rockefeller Institute) was used as the S-producing or type-specific strain. It was highly virulent, its lethal dose for mice never being greater than one-millionth cc. of culture. *Non-S-Producing Strain.*—The non-S-producing or non-type-specific strain was derived from the above type-specific strain by repeated transfers in broth made at infrequent intervals. The culture finally used had passed through 50 successive transfers in broth at intervals of 2 to 3 days; it had completely lost its S-producing function, and was no longer pathogenic for mice.

Serological Reactions of the S-Producing and Non-S-Producing Strains of Pneumococci.

In type-specific antipneumococcus serum, the S-producing strain exhibited the usual serological reactions; the bacteria themselves were agglutinated and the carbohydrate or S substance in culture filtrates was precipitated. In contrast, the bacterial cells of the non-S-producing strain were not agglutinated, nor were the supernatants of its broth cultures precipitated. The results obtained in species-specific antipneumococcus protein serum were quite different. The S-producing strain was not specifically agglutinated, nor was the carbohydrate substance in its filtrates precipitated. The non-S-producing strain, on the other hand, was agglutinated by the serum which contained the species-specific antiprotein antibodies.

The differences between the serological reactions of the S-producing and non-S-producing strains are essentially the same as those which have been reported by Reimann. The differences in agglutinability can be explained upon the basis of Avery and Heidelberger's schematic interpretation of the structure of the pneumococcus cell. The type-specific agglutination of the S-producing strain may be

[1] The term "S-producing" strain as employed throughout this paper refers to the original, virulent, highly type-specific strain which possesses the specialized cellular function of elaborating the immunologically reactive carbohydrate substance. The same properties are exhibited by Reimann's "S" strains.

The term "non-S-producing" strain refers to the avirulent, non-type-specific strain which has lost the property of producing the soluble carbohydrate. Its properties are similar to those of Reimann's "degraded" or "R" strains.

referred to the reaction between the ectoplasmic layer of specific carbohydrate and the type-specific anticarbohydrate antibodies; the species-specific agglutination of the non-S-producing strain may be referred to the reaction between the protein on the outside layer of the degraded bacterial cell and the species-specific antiprotein antibodies.

Antigenic Properties of the S-Producing and Non-S-Producing Strains of Pneumococci.

The differences in the antibody-invoking properties of the S-producing and non-S-producing strains were investigated by immunizing two series of rabbits with heated suspensions of the two sorts of pneumococci. The production of test-tube demonstrable antibodies was essentially the same as that reported by Reimann (5). The S-producing strain invoked the production of anticarbohydrate antibodies (type-specific agglutinins and type-specific S precipitins). The non-S-producing strain, on the other hand, was entirely devoid of the capacity to invoke the anticarbohydrate antibodies, and the serum obtained contained only the species-specific antiprotein antibodies and none of the type-specific antibodies.

The differences in the protective value of the serum yielded by immunization with the two strains are of more importance in the present paper than are the above serological reactions. The serum of animals immunized with the S-producing strain had a high passive protective value, regularly protecting mice against 0.01 cc. of virulent Type II pneumococci. In contrast, the serum of all animals immunized with the non-S-producing strain was entirely devoid of any protective power, although it possessed a high titre of species-specific antiprotein antibodies. Apparently, when the Type II pneumococcus cell loses its S-producing function, it no longer possesses the antigenic complex which invokes the production of passively protecting antibodies.

Active Immunization of Mice with S-Producing and Non-S-Producing Pneumococci.

Since there are frequent differences between passive and active antibacterial immunity (8), the following experiments were made to determine whether or not active immunity could be established by the vaccination of mice with non-S-producing pneumococci.

One series of ten mice was immunized with heat-killed vaccine prepared from the type-specific or S-producing strain, and another series with vaccine from the non-S-producing strain of pneumococci. Three courses of subcutaneous injec-

tions were given with a rest period of 1 week between courses; each course consisted of five doses of vaccine, each dose being given at 2 day intervals, equivalent to 0.15 cc. of broth culture. Tests for active immunity were made 11 days after the last vaccination, by the intraperitoneal injection of different amounts of Type II pneumococcus culture (10^{-6} to 10^{-3} cc.). Normal mice (not vaccinated) were injected with 10^{-6} cc. as virulence controls of the culture.

The results of the active immunity tests revealed the same differences in the antigenic properties of the two strains as those previously observed in the passive protection experiments. Although the protection was not uniform, some of the mice which had been actively immunized with the S-producing pneumococci survived the injection of doses of virulent Type II organisms equivalent to as much as one-thousandth cc. of culture.[2] In contrast to the series immunized with the type-specific organisms, none of the animals which had been vaccinated with the non-S-producing strain had acquired any detectable immunity and all were infected by doses of one-millionth cc. of the virulent culture. Apparently, therefore, the loss of the S-producing function of Type II pneumococci was accompanied by loss of the capacity to establish active immunity in mice, as well as by loss of the capacity to invoke passively protecting antibodies.

Absorption of Type II Antipneumococcus Serum with S-Producing and Non-S-Producing Pneumococcus Cells.

The results of preceding experiments have shown distinct differences in the antibody-invoking properties of the S-producing and non-S-producing pneumococci: the serum of animals immunized with the S-producing strain contained type-specific antibodies (agglutinins and S precipitins) and passively protected mice against Type II pneumococcus infection; in contrast, the serum of animals immunized with the non-S-producing strain contained no demonstrable type-specific anti-

[2] The irregularity in the protection of the mice in this series of vaccinated mice had no relation to the dose of pneumococci injected in the active immunity tests. Some individuals injected with 10^{-6} cc. and 10^{-5} cc. were infected, while others injected with 10^{-4} cc. and 10^{-3} cc. were protected. As reported in the following paper, the results of an investigation with a larger series of mice show that the above irregularities were due to differences in the immunity response of the individual mice.

bodies and did not protect mice. In view of these marked differences in the antibody-invoking properties of the two strains, experiments were made to determine if like differences existed in the capacity of the two strains to "absorb" or remove antibodies from type-specific immune serum.

A type-specific serum obtained by immunization of a rabbit with Type II pneumococcus vaccine was used in the absorption experiment. This serum contained the usual type-specific agglutinins, S precipitins, and passively protecting antibodies.

10 cc. of serum were placed in each of three tubes. 10 cc. of a salt solution suspension of heat-killed bacteria of the S-producing strain were added to one tube of serum; the same amount of a suspension of the non-S-producing pneumococci

TABLE I.

Absorption of Type II Antipneumococcus Serum with S-Producing and Non-S-Producing Pneumococci.

Type-specific antipneumococcus serum	Type II agglutinins	Precipitins for Type II soluble substance	Passively protecting antibodies
Unabsorbed	+	+	+
Absorbed with S-producing Type II pneumococci	0	0	0
Absorbed with non-S-producing pneumococci.	+	+	+

was added to the second tube of serum; an equal volume of salt solution was added to the third tube. The three mixtures were shaken for 5 minutes, then incubated for 2 hours at 37°C., and finally placed in the ice box for 12 hours. The tubes were then centrifuged to remove the bacteria. 10 cc. of the supernatant fluid from the above mixtures were then placed in each of three tubes and absorbed a second time by the same procedure.

After the removal of the bacteria used in the second absorption, the three sera ((1) unabsorbed control, (2) absorbed with S-producing pneumococci, (3) absorbed with non-S-producing pneumococci) were then tested for the presence of type-specific agglutinins, type-specific S precipitins, and type-specific protective antibodies. The agglutination and protection tests were made by the usual procedure. In the S precipitin tests, the bacteria-free filtrate of a young unautolyzed culture of Type II pneumococci was substituted for a solution of the purified carbohydrate.

The results of the experiment are summarized in Table I.

The results of the absorption experiments (Table I) reveal the same general immunological differences as those obtained in the preceding

immunization experiments. Absorption with the heat-killed cells of the S-producing strain removes all the S precipitins, type-specific agglutinins, and protective antibodies, just as immunization with the bacterial cells of this strain invokes the production of these same type-specific antibodies. On the other hand, absorption with cells of the strain in which the S-producing function has been lost fails to remove any of these antibodies, just as immunization with cells of this strain failed to stimulate the production of type-specific antibodies. Hence, the pneumococcus cells which have lost their S-producing function have lost not only their property of invoking type-specific antibodies by antigenic stimulation in the animal body, but have also lost their capacity to "absorb" the same antibodies by type-specific antigen-antibody combinations in the test-tube.

Absorption of Type II Antipneumococcus Serum with Culture Filtrates of S-Producing and Non-S-Producing Pneumococci.

In the following experiment, Type II antipneumococcus serum was absorbed with filtrates of young broth cultures of the S-producing and non-S-producing pneumococci. It seemed that the results to be obtained by absorption of the serum with the filtrates should be comparable to those obtained by use of solutions of the chemically purified carbohydrate since the S substance is the only immunologically reactive substance which can be demonstrated in filtrates of young and unautolyzed broth cultures of Pneumococcus. (The S substance is liberated into the culture fluid in the early period of growth and if the fluids are filtered before cell disintegration has commenced, the filtrates are entirely free of any of the bacterial protein or other serologically reactive substances.) To obtain fluids containing the maximum amount of the soluble substance, the cultures were planted in glucose broth. The reaction of the cultures was observed closely and when a pH of approximately 6.5 was reached, the bacteria were removed by centrifugation, the reaction adjusted to pH 7.3, and the fluids filtered through a Berkefeld candle.

5 cc. of the antipneumococcus (Type II) serum were then placed in each of three tubes. 15 cc. of the filtrate from the S-producing culture were added to the first tube; 15 cc. of the filtrate from the non-S-producing culture were added to the second tube; an equal volume of broth was added to the third tube as a control. All the mixtures were shaken for 15 minutes, incubated for 3 hours at 37°C., and then placed in the ice box for 18 hours. The mixtures were then centrifuged and the clear supernatant fluids removed. A second absorption similar to the above was carried out with 10 cc. of the supernatant fluid of each of the above serum mixtures. Only slight precipitation occurred when the S-containing filtrate was

added to the serum which had received the preceding absorption treatment with this filtrate. After 3 hours at 37°C. and 18 hours at 5°C., the second series of mixtures was centrifuged and the supernatant fluids again removed. The supernatant fluids were then subjected to a third absorption by the described procedure. The mixtures were again centrifuged and the supernatant fluids removed. The supernatant fluids of the third absorption series were employed in the following tests. The sera at this stage had been diluted to about one-twenty-fifth of their volume, so that each cc. of the test sera contained approximately 0.04 cc. of serum and 0.96 cc. of broth, or culture filtrate.

Tests for type-specific agglutinins were made by the usual method with dilutions equivalent to 1/25 and 1/100 of the original serum. Two series of passive protection tests were made with different amounts of serum. One series of mice

TABLE II.

Absorption of Type II Antipneumococcus Serum with Culture Filtrates of S-Producing and Non-S-Producing Pneumococci.

Type II antipneumococcus serum	Passive protection					Type-specific agglutinins	
	Mice injected with Type II pneumococci					Serum dilutions	
	Amount of culture						
	10^{-2} cc.	10^{-3} cc.	10^{-4} cc.	10^{-5} cc.	10^{-6} cc.	1/25	1/100
Unabsorbed control	S	S	S	S	S	+	+
Absorbed with culture filtrate of S-producing pneumococci	D	D	D	D	D	0	0
Absorbed with culture filtrate of non-S-producing pneumococci	S	S	S	S	S	+	+

S = survived 7 days. D = died 24 to 48 hours after injection.

received absorbed serum equivalent to 0.04 cc. of the original serum mixed with increments of broth culture ranging from 10^{-2} cc. to 10^{-6} cc. A second series of mice received the equivalent of 0.01 cc. of serum mixed with amounts of broth culture ranging from 10^{-3} cc. to 10^{-6} cc. The second series of protection tests was included to detect any incomplete absorption of protective antibodies which might not be recognized in tests with an excess of serum. Since the tests with the two amounts of serum gave identical results, only the results of the series with 0.04 cc. of serum are included in the summarized protocol presented in Table II.

Since the S substance was used for the absorption in the form of a solution in the filtrate of the S-producing strain, a slight excess of the carbohydrate remained in the serum mixture after the final absorption. Thus, it is obvious that a certain amount of the free carbohydrate was injected into the animals in the protection

tests with absorbed serum. In the absence of any evidence of an "aggressin"-like effect of small amounts of the S substance, we do not believe that this complicated the results of the experiment.

This experiment (Table II) shows that absorption with the filtrate of the S-producing strain removed all the type-specific agglutinins and also all the protective antibodies,[3] while the filtrate of the non-S-producing strain failed entirely to remove any of the type-specific antibodies and did not diminish the protective value of the serum. These results of the absorption with the culture filtrates were exactly analogous to the results (Table I) of the absorption with the heated whole cells of the two strains of pneumococci and, because of the simpler nature of the culture filtrates, furnish more direct evidence of the relation of Type II anti-S antibodies to protection.

The above results are also of interest from the standpoint of the identity of the type-specific agglutinin and the anti-S precipitin. Avery and Heidelberger's (3) contention that S precipitation and type-specific agglutination involve the same antigen-antibody combination, is supported by the fact that S precipitins are removed by absorption with suspensions of washed pneumococcus cells (Table I) and conversely by the fact that the type-specific agglutinins are removed by absorption with solutions of the S substance (Table II).

The studies of the soluble substance of Pneumococcus by Avery and Heidelberger (2, 3) and of the similar products of other bacteria by Zinsser and Mueller (9) have presented new conceptions of the importance of carbohydrates in immunology. From this point of view, the results in Table II are of general interest as an example of the removal of the protective antibodies of an antibacterial serum by absorption with solutions in which the only reactive substance is carbohydrate in nature.

DISCUSSION.

The elaboration of the soluble specific substance (carbohydrate) is a specialized function of the pneumococcus cell which is most highly

[3] It is important to note that this complete removal of protective antibodies by absorption with the S-containing fluid occurred in Type II immune rabbit serum. In similar experiments with Type I serum from an immune horse, protective antibodies were not completely absorbed when treated with culture fluid containing the Type I reactive carbohydrate.

developed in virulent strains. The loss of the function of S production is accompanied by changes in the immunological properties of pneumococci. As previously pointed out by Reimann (5), strains of pneumococci which no longer elaborate the specific carbohydrate, lose their type-specific serological properties and exhibit antigenic properties similar to those of solutions of pneumococcus protein (6, 7). In addition to the immunological changes manifested by differences in test-tube serological reactions, the loss of the S-producing function was also accompanied by marked changes in the antigenic properties involved in both passive and active protection of mice. The injection of S-producing pneumococci stimulated the production of passively protecting antibodies in rabbits and also established active immunity in mice. On the other hand, the similar injection of non-S-producing pneumococci neither invoked the production of passively protecting antibodies nor established active immunity.

The intimate relation of the type-specific antibodies to the antipneumococcus protection of mice was likewise evident in the results of absorption experiments. Absorption of Type II antipneumococcus serum with the heat-killed cells of S-producing pneumococci of homologous type removed the commonly recognized antibodies concerned in type-specific agglutination and in type-specific precipitation of solutions of the S substance. Serum from which these type-specific antibodies had been removed were completely devoid of passively protecting antibodies. On the other hand, when the Type II serum was subjected to the same absorption treatment with non-S-producing pneumococci, there was no detectable loss in the type-specific antibodies responsible for agglutination and S precipitation. This absorbed serum from which the type-specific antibodies had not been removed, possessed the same protective value as unabsorbed serum. More direct evidence of the relationship between the anticarbohydrate and the protective antibodies was furnished by the fact that absorption of Type II antipneumococcus serum with culture filtrates containing the S substance in solution removed the protective antibodies as completely as did the whole bacteria themselves. It is reasonable to believe that the removal of the protective antibodies was due to the reaction between the carbohydrate S substance and its type-specific antibody.

Although antipneumococcus immunity is type-specific and consequently the antibodies that confer it must also be type-specific, it is hazardous to conclude that Type II antipneumococcus protection is simply a function of the anticarbohydrate antibody. A number of reports have been made of the protective value of antipneumococcus serum which possesses none of the usual test-tube demonstrable antibodies. However, all our results indicate that the type-specific anticarbohydrate antibody is at least the dominant one involved in the protection of mice against virulent Type II pneumococci.

It is common experience (10) to find that the production of effective antipneumococcus serum requires the use of virulent pneumococci in the immunization. If the anti-S antibody is the one most prominently involved in Type II passive protection, the failure to obtain an effectively protecting serum by immunization with avirulent pneumococci would be explained not by the loss of the virulence of the bacteria employed as antigens, but by the fact that the avirulent bacteria no longer possess the antigenic complex (*SP* of Avery and Heidelberger (3)) which is required for the immunological production of type-specific antibodies.

SUMMARY.

The loss of the specialized function of S production by Type II pneumococcus was accompanied by a loss of the antigenic properties involved in both active and passive protection of mice. Absorption of Type II serum with S-producing pneumococci removed all the protective antibodies, as well as the type-specific agglutinins and S precipitins. The same absorption treatment of the serum by non-S-producing pneumococci failed entirely to remove the type-specific antibodies and did not affect the protective value of the serum. Absorption with bacteria-free culture fluids containing the reactive carbohydrate removed the protective antibodies as completely as absorption with the whole bacterial cells of type-specific strains. The results taken as a whole indicate that the antibodies involved in the usual protection of mice against Type II pneumococci are closely related, if not identical, to the specific anticarbohydrate precipitin.

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FACTORS INVOLVED IN THE INFECTION OF MICE AFTER VACCINATION WITH TYPE II PNEUMOCOCCI.

By JAMES M. NEILL, Ph.D., and EMIDIO L. GASPARI.*

(From the Department of Bacteriology and Immunology, Vanderbilt University Medical School, Nashville.)

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In a previous study[1] it was found that vaccination of a group of mice did not protect all the individual animals against subsequent infection with Type II pneumococci. The protection obtained was irregular; some individuals resisted relatively large numbers of pneumococci, while other individuals succumbed to much smaller doses. The present paper deals with a further study of the infection of individual mice after vaccination against Type II pneumococci. The experiments were planned to determine the relative importance of two factors in the postvaccination infections: the previous immunity response of the individual animals, and the dosage of the invading bacteria. The factor of differences in the immunity response of the individual is made especially prominent, for the animals (mice) not only possess no demonstrable natural immunity to the specific bacteria (pneumococci) but the vaccination was done with a type of Pneumococcus known not to produce a highly effective antibacterial serum.

EXPERIMENTAL.

Methods.

Immunization.—70 line-bred females, between 3 and 4 months old, which had been separated from males since weaning were selected for immunization.

The organisms from a 10 hour broth culture of Type II pneumococci (Strain D_{39}, Hospital of The Rockefeller Institute) were resuspended in salt solution and heated at 60°C. for 30 minutes. Each mouse received, subcutaneously, 0.5 cc. of a

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[1] Gaspari, E. L., Fleming, W. L., and Neill, J. M., *J. Exp. Med.*, 1927, xlvi, 101.

diluted suspension of the vaccine (equivalent to 0.1 cc. of broth culture) every 2 days for six doses. 10 days after the last injection a number of mice were tested for acquired immunity. The remainder were given a second course of immunizations with freshly prepared vaccine at 2 day intervals for five doses. After a rest period of 10 days, a number of animals were tested for immunity and the remainder were given a third course of immunizations with fresh vaccine exactly as in the second course; these animals were also tested for acquired immunity 10 days after the last injection.

A number of mice died during the period of immunization, and others were eliminated from the tests because of evident poor condition.

Tests of Immunity of the Animals (Protection Tests).—The tests of immunity of the vaccinated animals were made by intraperitoneal injections of 0.5 cc. of broth containing the desired amount of a 10 hour broth culture of the same virulent strain as that from which the vaccine had been prepared.

Experiments 1, 2, and 3: Influence of Size of Dose upon Infection: Active Immunity Tests of Vaccinated Mice by Injection of Different Numbers of Bacteria.

The first series of experiments (Experiments 1, 2, and 3) consisted of tests of the influence of size of dose upon the occurrence of infections in groups of mice which had previously received vaccination against Type II pneumococci. The doses used in these active immunity tests were 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} cc. of broth culture of pneumococci; this range of doses includes the numbers of pneumococci against which passive protection is usually effective. The mice used in Experiment 1 had received one course of vaccination before the immunity tests; the animals used in Experiments 2 and 3 had received, respectively, two and three courses. The purposes of the investigation are met by presenting the three experiments collectively, since each animal had received the same immunization treatment as the other individuals used in the same experiment. The results of of Experiments 1, 2, and 3 are presented in Table I; a summary of these results is collected in Table II.

Injections and Virulence Controls.—The vaccinated mice were injected as described under "Methods." The normal mice which were injected for virulence controls invariably succumbed to all amounts of culture tested—*i.e.*, from 10^{-7} cc. to 10^{-3} cc. The results of the virulence controls are presented collectively in the last column of Table I.

Control of Possible Effect of Weight of Individual Animals.—All the mice used in

TABLE I.

Tests of the Active Immunity of Vaccinated Mice.

Amount culture injected	Experiment 1. Immunity tests on first group of mice (after one course of vaccinations)	Experiment 2. Immunity tests on second group of mice (after two courses of vaccinations)	Experiment 3. Immunity tests on third group of mice (after three courses of vaccinations)	Virulence controls* (non-immunized mice) on Experiments 1, 2, 3
cc.				
10^{-3}	D 24–48 hrs. S 5 days	D 24–48 hrs. D 24–48 hrs. D 24–48 hrs.	D 24–48 hrs. D 24–48 hrs. D 24–48 hrs. D 24–48 hrs. D 48–72 hrs. S 5 days S 5 days	D 24–72 hrs. D 24–72 hrs.
10^{-4}	D 24–48 hrs. S 5 days	D 48–72 hrs. S 5 days S 5 days	D 48–72 hrs. D 48–72 hrs. D 48–72 hrs. D 48–72 hrs. D 48–72 hrs. D 48–72 hrs. S 5 days	D 24–72 hrs. D 24–72 hrs.
10^{-5}	D 24–48 hrs. S 5 days	D 24–48 hrs. D 24–48 hrs. S 5 days	D 24–48 hrs. D 48–72 hrs. D 48–72 hrs. D 48–72 hrs. D 48–72 hrs. S 5 days S 5 days	D 24–72 hrs. D 24–72 hrs.
10^{-6}	D 24–48 hrs. S 5 days	S 5 days S 5 days S 5 days	D 24–48 hrs. D 24–48 hrs. D 24–48 hrs. D 48–72 hrs. D 48–72 hrs. D 48–72 hrs. S 5 days	D 24–72 hrs. D 24–72 hrs.
10^{-7}	No tests	No tests	No tests	D 24–72 hrs. D 24–72 hrs.

D = died of pneumococcus septicemia.

S = survived.

* Presented in composite form; duplicate mice were injected with each amount of culture in each of the three experiments.

the experiments were in excellent physical condition but varied in weight from 24 to 31 gm. To rule out any possible influence of weight upon the resistance to infection, the mice of different weights in Experiments 2 and 3 were matched so that the tests of the different amounts of culture were made with a series of animals of comparable weight. The results showed that there was no correlation between weight of the animal and its susceptibility.

Survival in Table I.—Since all the virulence controls died in less than 72 hours after injection the protection of the vaccinated mice is based upon survival for 5 days after the test injection. Three of the mice reported as "Survived" in Table I died between 10 and 14 days after injection.

The most obvious fact in Table I is the irregularity in the survival and death of the vaccinated mice. In each of the three experiments individual mice were infected by doses smaller than the doses which

TABLE II.

Summary of Tests for Active Immunity of All the Vaccinated Mice: Proportion of Animals Protected against Different Doses of Pneumococci.

Amount of culture injected.	Number of vaccinated mice tested	Number of mice protected	Proportion of mice protected
cc.			*per cent*
10^{-3}	12	3	25
10^{-4}	12	4	33
10^{-5}	12	4	33
10^{-6}	12	5	42
10^{-3} to 10^{-6}	48	16	33

other mice survived and *vice versa*. This lack of relationship between the number of pneumococci injected and the infection of the vaccinated animals is emphasized in the summary of the experiments given in Table II. There it is seen that about the same proportion of the vaccinated mice survived the injection of each of the different doses; in fact, any deviation from the average proportion of one-third survivors represents the survival or death of only one mouse.

Since these facts indicate that within this range of dosage (10^{-6} to 10^{-3} cc.) the size of the dose does not influence the infection of the vaccinated animals, the infection or resistance of individuals would logically be attributed to differences in individual immunity. In these experiments the immunity of the animals was limited to that

acquired by vaccination. Therefore, within the range of dosage used in these experiments, the previous immunity response of the individual appeared to be the factor determining the postvaccination infections.

Obtaining a "Selected Group" of Actively Immune Mice.

The preceding results indicated that only a certain proportion of the mice respond effectively to pneumococcus (Type II) vaccine. This fact made it impossible to carry out additional experiments until we obtained a selected group of vaccinated mice. It seemed that a "selected group" of individuals known to possess an effective active immunity could be obtained by collecting all the mice which had survived the previous injections of live bacteria in Experiments 1, 2, and 3. These mice were given the following additional vaccination treatment.

Survivors of Experiment 1A.—10 days after the injection made in Experiment 1, all the surviving mice were put upon a vaccination schedule similar to that described under "Methods;" in addition to the immunization preceding the tests of Experiment 1, they received three vaccination courses of five injections each. *Survivors of Experiment 2A.*—These mice received, in addition to the immunization preceding the tests of Experiment 2, two courses of the described vaccination. *Survivors of Experiment 3A.*—These received one additional course of vaccinations. A few mice died during the immunization period.

These mice represent a "selected group" of animals known by previous tests to possess some degree of active immunity against Type II pneumococci and were used as described in the following experiments.

Occurrence of True Mass Infection in Actively Immune Mice.

It is an established fact that no amount of antipneumococcus serum can passively protect mice against overwhelmingly large numbers of virulent pneumococci. This phenomenon can properly be termed "true" mass infection, in that the passive immunity furnished by the antibacterial serum is known to be sufficient to protect against infection by smaller numbers of the bacteria. Experiments were made to determine whether active immunity likewise fails when the vaccinated animal is invaded by doses beyond the zone within which passive protection is effective. In view of the previously demonstrated differences in the response of individual mice to vaccination, the tests for "mass infection" were limited to animals known to have acquired sufficient active immunity to resist invasion by smaller numbers of pneumococci.

Four mice from the previously described "selected group" of actively immune mice were injected with 0.02 cc. of broth culture of Type II pneumococci. Although these mice possessed a fairly high degree of antipneumococcus active immunity, they all died of pneumococcus septicemia. (That all of them possessed

at that time sufficient immunity to protect them against at least 10^{-4} cc. of broth culture is shown by the fact that all other individuals of the "selected" group survived that dosage when tested in a simultaneous experiment.)

The results of this experiment showed that, even in a selected group of actively immune mice, infection invariably follows invasion by overwhelmingly large numbers of pneumococci regardless of the previous immunity response of the individual. This experiment presents a definitely controlled example of the true mass infection of actively immunized animals, in that the infected individuals were known to possess sufficient immunity to protect them against smaller numbers of pneumococci.

Comparison of the Occurrence of Infections in an "Unselected" and in a "Selected" Group of Vaccinated Mice When All the Individuals Are Injected with the Same Number of Pneumococci (Type II).

The importance of the factor of individual immunity response in postvaccination infection was further illustrated by the following comparison of the percentage of infections which occur when a constant number of bacteria is injected into two different groups of vaccinated mice.

In one group, the factor of differences in individual immunity response was eliminated by including only animals known by previous test to have sufficient immunity to resist the invasion of at least a small number of pneumococci; in the other group, this factor was uncontrolled (as in Experiments 1, 2, and 3), and although all the animals had received the same vaccinations, no previous tests had been made to determine the effectiveness of their immunity response. The same number of pneumococci was injected into each individual animal; the dose employed (10^{-4} cc. of broth culture) was below the zone of true mass infection.

The tests were carried out with the following three groups of mice:

1. The "selected" group of vaccinated animals which had been found in previous tests to have sufficient active immunity to resist infection against at least a small number of Type II pneumococci. These mice had received the vaccination treatment indicated in the preceding description of the procedure employed in obtaining the "selected population." (In addition to the heat-killed vaccine,

they had received an injection of living bacteria in connection with Experiment 1, 2, or 3.)

Five mice from this group were inoculated with the test dose.

2. The "unselected" group of vaccinated animals which, although vaccinated, had never been tested to see whether or not they had developed any immunity. (This group is analogous in this respect to the animals used in Experiments 1, 2, and 3.) The routine of immunization was the same as that described previously under "Methods," with the exception that the mice in this group received one additional course of vaccinations.

Nine mice from this group were inoculated with the test dose.

3. A group of normal animals was included to furnish evidence of the invariability of infection of non-vaccinated mice when invaded by the test dose of

TABLE III.

Occurrence of Infection in a "Selected" and "Unselected" Group of Vaccinated Mice When Injected with the Same Dose of Type II Pneumococci.

Groups of mice tested		Number of animals injected	Number survived	Percentage of animals protected
Vaccinated mice	"Selected" group (individuals previously tested for acquired immunity)	5	5	100
	"Unselected" group (individuals not previously tested for acquired immunity)	9	2	22
Normal (not vaccinated) mice	"Selected" group (animals possessing no natural immunity)	6	0	0

bacteria. Six mice received the test dose of 10^{-4} cc.; in addition, the usual duplicate virulence controls were made with 10^{-5}, 10^{-6}, 10^{-7} cc. of culture.

The results of the experiment are summarized in Table III.

The results of this experiment (Table III) furnish further evidence of the importance of the previous immunity response of the individual in the determination of the occurrence of the postvaccination infections. The two groups of vaccinated mice, although they had received approximately the same previous immunization treatment, represent respectively, an "unselected" and a "selected population." In the "selected" group where the factor of marked differences in individual immunity response had been eliminated, the protection was regular and no infections occurred. On the other

hand, in the "unselected" group (where the actual immunity response of the vaccinated animals had not been tested) some mice were infected and some were not, in spite of the fact that each individual was known to have been invaded by the same, reasonably small number of bacteria. Whether or not infection occurred in the "unselected" group probably depended upon the response of the respective individuals to the previous vaccination.

The chief interest of these results is due to the fact that two factors were controlled which are impossible to control in bacterial infections outside the laboratory: (1) dosage or numbers of the invading bacteria; (2) differences in individual immunity response to the vaccination. When the first of these two factors is constant, the occurrence of the infection depends upon the second factor, and the percentage of infection within the group depends upon the percentage of the individuals that had previously responded effectively to the vaccination. When the second factor is kept constant (as in the "selected population" of known immunes) the occurrence of infection depends upon the numbers of bacteria, and all the individuals are protected if the dosage is kept below the zone of true mass infection.

Type Specificity of Active Immunity of Mice Vaccinated with Type II Pneumococci.

The type specificity of the active immunity of mice vaccinated with Type II pneumococci was tested as follows: Four mice were taken from the described "selected population" of Type II-immune individuals and injected respectively with one of the following amounts of virulent Type I organisms, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} cc. of broth culture. (That the animals actually possessed active immunity toward the homologous pneumococci (Type II) was controlled by simultaneous tests of the homologous protection of other individuals from this "selected" group of mice.) Duplicate normal animals were injected with the same amounts to serve as virulence controls of the culture.

The mice which possessed a high degree of active immunity to Type II pneumococci died at approximately the same time as did the normal (unvaccinated) animals, and thus showed no evidence of immunity against the heterologous (Type I) pneumococci. This experiment is of interest as evidence that antipneumococcus (Type II) active immunity of mice is just as type-specific as the immunity in passive protection tests.

DISCUSSION.

The preceding experiments have dealt with the relative value of dosage and individual immunity response as factors in the infection of mice which had previously been vaccinated with Type II pneumococci. After the completion of vaccination the resistance of the individuals was tested by the intraperitoneal injection of different doses of live bacteria. The results of the tests of active immunity can be analyzed to best advantage according to two zones of dosage.

In the first zone (between 10^{-6} and 10^{-3} cc. of broth culture) the infection or protection of the vaccinated animals was irregular, and certain individuals succumbed to doses much smaller than those which other individuals resisted. The differences in the number of live bacteria injected within this zone seemed to be entirely without influence in the determination of infection or protection since the percentage occurrence of infection was approximately the same in the groups injected with each of the different doses although the maximum dose (10^{-3} cc.) was 1000 times the minimum dose (10^{-6} cc.) employed in the experiments. This apparent absence of any relation between size of dose and infection indicates that the irregularities in infection and protection are due to differences in the immunity of the individuals. Since the mice possessed no demonstrable natural immunity to the specific bacteria, the differences in the degree acquired must be referred to differences in individual response to the same vaccination. Hence, in this zone of dosage (10^{-6} to 10^{-3} cc. of culture), the occurrence of postvaccination infection was determined by the lack of previous immunity response of the individual rather than by the numbers of the invading bacteria.

In the second zone of dosage, the relations were reversed and the injection of overwhelming numbers of the bacteria invariably resulted in infection in spite of the fact that the animals were known to possess a fairly high degree of active immunity. The infection of the mice tested in these controlled experiments can properly be termed true "mass infection;" but it is well to recognize that the same term is often applied to postvaccination infections where the actual immunity of the individual is as unknown as is the dosage.

The above demonstration of the relative value of these two factors

in the infection of mice after vaccination with Type II pneumococci is of some interest in the question of postvaccination infections in general. In our experiments, the factor of differences in individual response to the vaccination was made especially prominent by choosing animals possessing no demonstrable natural immunity and vaccinating them with a type of Pneumococcus known not to give a high degree of passive immunity. However, the same factor is probably involved, if much less prominently, in the effectiveness of the vaccination of other animals against other bacteria. The failure of one attack of typhoid fever to render certain individuals immune must be evidence of differences in the immunity response of men to typhoid bacilli. Variations in the passive protective value of convalescent serum from pneumonia and other diseases probably represent examples of the same phenomenon.

SUMMARY.

A group of mice was vaccinated against Type II pneumoccci and subsequently tested for immunity against different numbers of the live bacteria. The immunity tests were conducted within two zones of dosage. In the first zone where the doses were kept within reasonable limits (10^{-6} to 10^{-3} cc. of culture), the number of invading bacteria was without influence and the occurrence of infections was determined by the previous immunity response of the individual. In the second zone of dosage (where passive protection also fails), these relations were reversed, and invasion by overwhelming numbers of the bacteria invariably produced infection regardless of the previous immunity response of the individual.

These results present an extreme example of the importance of the immunity response of the individual as a factor always concerned in the effectiveness of vaccination.

STUDIES ON PATHOGENIC B. COLI FROM BOVINE SOURCES.

I. The Pathogenic Action of Culture Filtrates.

By THEOBALD SMITH, M.D., and RALPH B. LITTLE, V.M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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The strains used in these studies were all isolated from the ileum of young calves passing liquid feces and showing signs of a choleriform disease (scours). Most of the strains were obtained from sick calves, some moribund, immediately after they had been killed, a few after natural death. Several strains from normal calves were introduced for comparative study. They differ from one another in motility, fermentative capacities, the possession of capsules, tendency to mutate, and the like. More detailed descriptions will be given in papers to follow.

B. coli, representing a group, may be regarded as a special type of parasite, restricted to the digestive tube with occasional sallies into those organs or cavities where the protective forces of the host cannot be fully exerted, as in the urinary tract. It was thought that any new light which can be shed on its behavior might be useful in interpreting the behavior of the more highly specialized and parasitic paratyphoid group.

In various articles one of us has formulated the hypothesis that in the gradual evolution of pathogenic or invasive types of bacteria, the beginnings of parasitism may have been made possible by a soluble, diffusible toxin, but that in later stages this primary offensive, more or less accidental, mechanism is either partly or wholly suppressed and some different mechanism developed with which the bacteria protect themselves against the body-foreign forces of the host. The process may be regarded as shifting from the destructive, predatory to the parasitic, from the offensive to the defensive type. According to

this hypothesis, *B. coli* represents the early predatory, toxic stage, with, however, a certain specialization towards protection from anti-foreign activities in the digestive tract. It resembles in many respects the cholera vibrio in its activities. In view of the hypothesis presented, a study of any early appearing toxin in cultures seemed the first problem to be attacked.

EXPERIMENTAL.

Effect of a Living Culture on Calves.—In the following pages, the effect of culture filtrates on calves is the chief topic. As a preliminary, the notes on the effect of a living bouillon culture are here introduced.

A calf (No. 307), 32 days old, weighing 104 lbs., received into a jugular vein 2 cc. of a 24 hour bouillon culture of *B. coli* 223. This strain produced a large amount of viscid material, even within 24 hours. In 2 days, the entire bouillon became viscid, so that short, cobweb-like threads could be raised from the fluid. The calf was seen 25 minutes later. When seen again, 65 minutes after the injection, it was lying on its side, dead, and a mass of white froth about 12 inches long extended from its nostrils on the floor of the stall. The autopsy showed the following conditions.

Digestive tract pale throughout. Upper respiratory tract, including pharynx and larynx, cyanotic. Tracheal mucosa injected and covered with froth. Lungs large and heavy. Both large caudal lobes are intensely and uniformly congested, the condition bordering on hemorrhage. The condition as to blood content varies from lobule to lobule. The smaller lobes (ventral, cephalic) are far less congested and edematous. Subendocardial tissue, left side, around papillary muscles, infiltrated with blood in form of large patches. Liver with borders rounded. Over large areas there are confluent patches of a dark red color. These, on section, correspond to diffuse hemorrhage into parenchyma similar to the pulmonary lesions. Both kidneys have large dark red areas in the cortex. This hemorrhagic condition dips down in a linear way to medulla. Besides these radiating lines of hemorrhage, hemorrhagic patches are present, as in liver and lungs. Urine from bladder clear, amber-colored, and free from any visible blood tint. It contains a small amount of coagulable protein. In sections of lung, kidney and liver the described lesions are shown to be due to an intense congestion or filling up of the capillary system associated with and merging into hemorrhage. When 16 days old this calf was fed with a heavy suspension of the same culture without showing disturbance of any kind.

Effect of Filtrates.—In a study of filtrates it was deemed best to restrict the experiments on calves to relatively young cultures. Veal broth containing 1 per cent peptone and 0.1 per cent dextrose was

sterilized in flasks in layers 2 to 3 cm. deep. 48 hours after inoculation the cultures were filtered through Berkefeld filters and the filtrate stored in full bottles at 38–40°F. until used. Any deterioration within several months was not observed.

The effect on calves 1 to 2 months old, on calves 6 to 7 months old, and on cows was qualitatively the same when the filtrate was injected into a jugular vein. Subcutaneous injection was without visible effect. After the intravenous injection of 2 cc. of filtrate the first signs in calves were manifest in from 5 to 20 minutes by a slight cough. After this there was a speedy increase in the number of respirations and pulse beats, the former rising to 100, rarely 120 or higher, the latter to 80 or even 100 and above. After 1 or more hours, both declined and the respirations became somewhat jerky. The expirations were usually interrupted and accentuated by a grunt. The temperature rose 1° or 2°C. after the injection but was normal the next day. The respiratory difficulties may in some animals become very great. The mouth is then held open, and saliva dripping from it, the head and neck held horizontal. Usually the calf is very restless, lies down and gets up repeatedly, or when very weak it lies on its side with the legs extended.

The reaction following the intravenous injection is, as might be supposed, not the same in all calves. A few failed to manifest the acute respiratory distress and the reaction showed itself in muscular tremors and chills. In most calves there were repeated discharges of semi-liquid feces in addition to the respiratory symptoms. The pronounced symptoms usually last 4 to 6 hours. Rarely the depression continues over 1 or 2 days. The following protocol is inserted to illustrate the time intervals of the several stages of the toxic effects.

10.20 a.m. Calf, 34 days old, Holstein female, receives, intravenously, 2 cc. filtrate of a 48 hour culture of *B. coli* 1085.

10.25 a.m. Calf very sick, lies down; respirations 130. Coughs frequently.

10.50 a.m. Respirations 88; pulse 60. Respiratory conditions the same.

11 a.m. Respirations 40. Temperature 38.9°C. Pulse 70.

12.15 p.m. Lying with head extended and legs straightened at right angles to body. An expiratory grunt as of some obstruction to expulsion of air.

3.15 p.m. Still lying in the same position. Temperature 39.1°C. A grunt with each expiration but not with any check in the movement. Respirations 70.

4 p.m. Temperature 39.4°C. Still lying down exhausted. Respiration as before. Copious discharge of feces.

5 p.m. Calf standing up. Brighter. Takes its evening food. Temperature 38.9°C.

Calf slightly depressed on the following day. Temperature about normal.

In calves 6 to 7 months old the same dose of 2 cc. produced severe reactions. One cow treated with the filtrates intravenously reacted severely after each injection of a dose increasing gradually to 15 cc. The filtrates of five strains of *B. coli* from calf scours distinguishable culturally from one another all produced the same succession of symptoms.

The mode of introduction of the filtrate naturally brings the respiratory tissues first under the influence of the toxin. The symptoms indicate an injury of the alveolar epithelium and vascular endothelium leading to increased permeability and transudation of fluid into the alveoli. That the toxin is a capillary poison is furthermore indicated by the lesions found in the fatal case following the injection of a living culture described above, and the following cases in which the autopsy showed the end effect on the lung tissue.

Holstein heifer calf, 48 days old, received into a jugular vein, a mixture of 2 cc. *B. coli* filtrate (1192b) and 6 cc. serum from a cow which had been treated with filtrate. This calf had received intravenously 2 cc. filtrate and 2 cc. serum when 38 days old. It went through the typical reaction associated with rapid respiration, open mouth and dribbling of saliva, 35 minutes after the injection. Dyspnea became pronounced and associated with a grunt during each expiration. The labored breathing continued from 11 a.m. until well into the night. The calf appeared free from any respiratory difficulties next morning. It was killed 2 days later. The viscera were normal with exception of the lungs which displayed an irregularly distributed congestion and small hemorrhagic areas. Sections showed the presence of deformed, cup-shaped red cells in small numbers in the alveoli of various regions. Distinct hemorrhagic areas were also present. In some lobules the alveoli contained granular and fibrillated material evidently fibrin. Polymorphs were loosely distributed in small numbers in the alveoli partly enmeshed in the alveolar coagulum. Mitoses of alveolar epithelium were not infrequent. This animal thus was still under the influence of a pulmonary congestion associated with small hemorrhages. There was no evidence of a persisting early or fetal pneumonia.

A Guernsey male calf, 4 weeks old, received into a jugular vein 2 cc. of a filtrate of *B. coli* 223, the same strain which had been fatal to Calf 307, 7 years ago. The filtrate was prepared from a bouillon culture 48 hours old.

The symptoms referable to the respiratory tract followed in the order described but with unusual intensity. The respiratory dyspnea came on within 30 minutes.

The calf breathed with mouth open. The respirations rose to 112, but dropped to 48 after 4 hours. At this time the calf was too weak to stand. The respiratory difficulty increased and the calf died about 5½ hours after the injection. The autopsy immediately after death showed besides a general congestion of the viscera, an intense congestion of the lungs. The right cephalic lobe was completely airless, heavy. On section the parenchyma was evidently filled with blood. The left cephalic lobe contained a little air. The interlobular tissue was broadened by hemorrhagic infiltration. The azygos lobe was in part airless through hemorrhages. Both large caudal lobes showed all degrees of congestion, edema, and hemorrhage from the still air-containing caudal tips to the cephalic margins of these lobes.

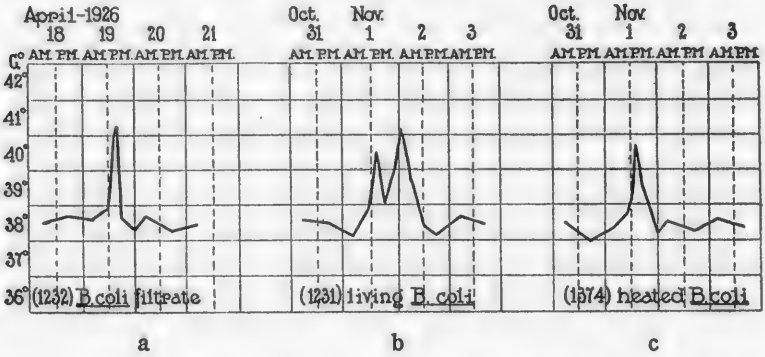
The spleen was enlarged through congestion. The kidneys were moderately congested, with a more deeply injected zone between cortex and medulla. Urine dribbling from the urethra during the early hours after injection of the filtrate was clear, slightly alkaline, specific gravity 1.002, with no protein coagulable by heat. Urine taken from the bladder soon after death had a specific gravity of 1.015, and contained coagulable protein equivalent to a deposit of 1.3 cc. in 10 cc. fluid.

Another calf received subcutaneously, at birth, 40 cc. serum from a cow treated with *B. coli* filtrates. When 29 days old and weighing about 112 pounds, it received into a jugular vein 2 cc. filtrate of a 48 hour bouillon culture of *B. coli* 1192a diluted with 3 cc. bouillon. The filtrate was 49 days old and it had been stored continuously at 36–40°F. in full bottles. Symptoms began within 20 minutes and ran the usual course with panting respirations rising to 120 per minute. The calf stood with head low, mouth open, and tongue protruding. 1½ hours after injection the respirations had fallen to 72. The animal was then very sick and unable to stand. Grunts with every expiration. 3½ hours after injection the animal began to be easier. Respirations 60, with occasional grunts. In 6 hours the reaction was nearly over and 1 hour later the evening meal of milk was taken readily.

The calf was killed about 23 hours after injection when in outwardly normal condition. The vessels of neck were severed after stunning the animal. The only organs visibly changed were the lungs. These were extensively involved. Three forms of lesions could be observed. (*a*) A dark red hepatization due to exudation into alveoli of blood and coagulation there. About 8 cm. of the free tip of the left cephalic lobe was in this condition. (*b*) Localized congestions and hemorrhages involving one or several lobules. These were scattered through the main (caudal) lobes chiefly. (*c*) Petechial hemorrhages 1 to 3 cm. apart, chiefly in the small cephalic lobes. The liver showed a patchy congestion visible both on surface and on section. Each liver cell contained one or more fat globules 2 to 5μ in diameter. Kidneys slightly congested and distinctly more moist on section than normal. Hyperemia of boundary zone between cortex and medulla. Spleen congested but normal markings still visible. Slight patchy hyperemia of upper small intestine. Urine about 30 minutes after injection contained about

1.3 per cent deposit of coagulable protein after heating. At autopsy there was only about 0.3 per cent. Urine 12 days before injection was normal.

Sections of fixed and hardened tissue presented nothing noteworthy beyond what is stated in the autopsy notes concerning the liver, spleen, and kidneys. In sections of the lungs, that portion found consolidated at autopsy presented various lesions. Focal hemorrhages were numerous and filled a group of contiguous alveoli with red cells. Forming a background for these hemorrhagic areas, the remainder of the alveoli contained a delicate network of fibrin fibrils holding enmeshed polymorphs in small numbers. The latter cells were brought together in denser groups within alveoli here and there. The other lobes of the lungs showed areas with partly collapsed alveoli and broadened walls as well as occasional small hemorrhages and alveolar fibrin. Polymorphs were thinly distributed throughout, both in the capillary bed and the alveolar lumina.



TEXT-FIG. 1. Temperature reactions in calves following the intravenous injection of *B. coli* and filtrates.

Thus far only filtrates of 48 hour bouillon cultures had been used. The presence of abundant toxin in a 24 hour culture was demonstrated on a calf 26 days old. After the intravenous injection of 2 cc. of the filtrate the sequence of symptoms already described appeared and with an intensity fully equal to that following the 48 hour culture filtrate. The calf was killed within 48 hours of the injection. The lungs still presented signs of the reaction in the form of subpleural hemorrhagic points and scattering congested lobules.

The effect of the intravenous injections on the rectal temperature of calves is shown in Text-fig. 1. The effect of a filtrate of a 48 hour bouillon culture is shown in (*a*). A similar unimodal curve (*c*) is pro-

duced by cultures heated at 60°C. for 30 minutes and therefore sterilized. In (*b*) the bimodal curve following the injection of living cultures may be due to immediate effects of the toxin followed by a temporary multiplication of the injected bacteria and hence a second dose of toxins.

The experiments reported were made with 24 and 48 hour culture filtrates. One test was made with a filtrate of a culture of 1192a incubated 8 days. The layer of bouillon in the flask was about 1 cm. deep. At the end of the incubation, the fluid was quite viscid and hence diluted with an equal volume of normal saline to facilitate passage through a Berkefeld filter.

4 cc. of the filtrate, containing 2 cc. of the original culture fluid, was injected into a jugular vein of a calf 34 days old. The symptoms followed one another as in the preceding cases but with much greater intensity and rapidity. The calf died in 3½ hours after the injection. There was complete hemorrhagic filling up of the entire left lung excepting a narrow margin of ventral and cephalic lobe. On section, the tissue was uniformly dark red, with reddish frothy fluid trickling off. The tissue was heavy but still resilient. The right lung, along median and dorsal region, was in the same condition; the lateral two-thirds of this lung was still partly air-containing with dark red areas in each lobule. Much foamy reddish fluid flowed from cut section of the pinkish regions. The trachea was filled with a reddish froth. There was a moderate congestion of the kidneys and some fat in the liver cells. The mucosa of intestines was only feebly reddened. The spleen was congested and weighed 460 gm.

This preliminary test clearly indicated a rise in the toxicity of the culture fluid due to longer incubation. Whether there is but one toxin involved or others superadded during the longer incubation remains unanswered for the present.

The effect of filtrates on guinea pigs introduced into the peritoneal cavity is relatively slight when compared with the serious effect on calves weighing about 100 pounds. The guinea pig weighing 350 to 600 gm. receiving the calf dose of 2 cc. into the peritoneal cavity reacts only with loss in weight as follows: Within 2 days there is a loss of 35 to 50 gm. in weight. Then there is a recovery so that in 7 days the original weight has not only been regained but added to by 10 to 15 gm. A small portion of this loss may be produced with bouillon alone. The injection of smaller doses, up to 0.5 cc., directly into the

heart failed to produce any acute symptoms or later death. In relation to body weight 0.5 cc. into the circulation of guinea pigs is over 30 times, the intraperitoneal dose of 2 cc. over 120 times the calf dose. In an early, fairly comprehensive study of calf scours, E. Joest[1] finding that filtrates of 24 hour bouillon cultures of *B. coli* were non-toxic for guinea pigs after intraperitoneal injection up to 3 cc., concludes that soluble poisonous products are not secreted.

It has already been stated that the subcutaneous injection of filtrates is without appreciable effect. The same is true when living cultures are introduced with the food. Two calves, about 2 months old, were fed *B. coli* in milk without showing any digestive or other disturbances. 500 cc. milk had been heavily seeded with a bouillon culture, warmed, and incubated for 7 hours. Plate cultures indicated ½ billion bacteria per cc. To disguise the flavor, fresh milk was added. The incubated milk coagulated when heated and was strongly acid to litmus.

Owing to obvious difficulties in multiplying experiments on calves only a few have been made bearing other phases. When bouillon cultures were shaken with kaolin and filtered there was no reduction of toxicity. When the filtrate was exposed to 80°C. for 30 minutes the toxicity though decidedly reduced was not completely destroyed.

One calf was treated with a 48 hour culture filtrate of a paratyphoid bacillus from guinea pigs.[2] 4 cc. in place of the usual 2 cc. were injected into a jugular vein. The symptoms following were similar to those produced by the *B. coli* filtrate, but less severe. The calf began to cough in 13 minutes. In 15 minutes the respirations were 104. In 30 minutes the calf was lying down, with respirations at 80 and evidently labored. After 1½ hours, respirations were 60 and dyspnea pronounced. Viscid saliva was hanging from the mouth. After 3 hours, the calf was lying quiet and apparently without distress. It took its milk after 6 hours. Next day it was still subdued. During the attack the temperature rose 1–1.5°C.

The soluble toxins of the large group of typhoid, paratyphoid, and colon bacilli have interested many observers since these groups were definitely recognized. Much of the work has been done in the paratyphoid group owing to its close relation to outbreaks of meat and

[1] Joest, E., *Z. Tiermed.*, 1903, vii, 377.

[2] Nelson, J. B., and Smith, T., *J. Exp. Med.*, 1927, xlv, 353; and Smith, T., and Nelson, J. B., *J. Exp. Med.*, 1927, xlv, 365.

other food poisoning. A fairly complete bibliography has been published recently by Miss Branham.[3] Among the recent papers which bear directly on the subject of this communication is one by Steinberg and Ecker[4] who prepared an antiserum in rabbits towards the soluble toxin with a culture fluid centrifuged but not filtered. The antiserum was tested upon rabbits inoculated with living cultures. The results apparently demonstrated the neutralizing power of the antiserum on the soluble toxin. An analysis of the experiments does not bear out the inference drawn. The rabbits received some living bacteria as antigen and the effect of the antiserum in the test rabbits may have been a suppression of the bacteria injected rather than a neutralization of the soluble toxin.

SUMMARY AND CONCLUSIONS.

The relatively young bouillon filtrates, 24 and 48 hours old, of certain strains of *B. coli* obtained directly from the ileum of scouring calves, were highly toxic for calves about 1 month old, as well as for older calves and cows when given into a vein. The symptoms, of panting followed by dyspneic and jerky respiration, indicate some at first obstructive action upon the alveolar and endothelial cells, followed by a greater permeability and eventual filling up of the air spaces with a serous, fibrinous, and hemorrhagic exudate. Similar effects are produced in other organs, such as liver and kidneys, if the toxin reaches them or is formed there by multiplying bacteria. There are no immediate or remote effects resembling those on calves following the intraperitoneal or the intracardiac injection of *B. coli* filtrates into guinea pigs even when the dose represents many multiples, per body weight, of the dangerous or even fatal calf dose.

The administration of the filtrate subcutaneously is without visible effect. Similarly, feeding large numbers of living bacilli produced no manifest disturbances.

In support of the hypothesis of a genetic relation between the group of *B. coli* and of paratyphoid, a similar but less severe effect was produced in a calf by the intravenous injection of a bouillon filtrate of a paratyphoid strain.

[3] Branham, S. E., *J. Infect. Dis.*, 1925, xxxvii, 291.

[4] Steinberg, B., and Ecker, E. E., *J. Exp. Med.*, 1926, xliii, 443.

STUDIES ON PATHOGENIC B. COLI FROM BOVINE SOURCES.

II. Mutations and Their Immunological Significance.

By THEOBALD SMITH, M.D., and GLADYS BRYANT.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

Plates 6 and 7.

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A prominent and early phenomenon presented by the colon types from the ileum in calf scours was the appearance of thin peripheral outgrowths from surface colonies on agar plates which represented a mutation of the colony nucleus. These outgrowths began to appear in the incubator within 2 days on plates from the original intestinal material. They also appeared regularly on plate cultures from later transfers. One of the conditions preventing their appearance is a crowding of the colonies. These must be at least $1\frac{1}{2}$ to 2 cm. apart to furnish the favorable environment. They form at incubator and room temperature.

Reproductions of the mutants which have thus far been more thoroughly studied from one or more angles are shown in Figs. 1 to 4 which may serve in place of detailed descriptions. It will be noted that two modes of formation of the outgrowths are presented. In one (Nos. 1127, 1085, 1192) they appear as rounded lobes or wings on the margin of the mother colony. This is thick, opaque, whereas the outgrowths are thin, partly translucent. As the lobes expand and the entire colony becomes enclosed in a zone of lobes the inner opaque colony becomes jagged and shrunken and often like an irregular cross in outline. The second form is shown in Fig. 4. The thin outgrowths appear as minute webs in notches of the original colony. These notches increase in width and depth and, as a result, the original colony has an outer zone of finger-like projections with the mutant outgrowth stretched between them. The tendency to form wing-like

expansions was not confined to one race. The strains figured differ from one another in one or more characters. Thus No. 223 is a motile, saccharose-fermenting strain; Nos. 1192, 1127, and 1085 are non-motile, non-saccharose-fermenting strains. All differ from one another in degrees of virulence.

The mutants obtainable on agar plates from lateral expansions of the colonies will be called (b) in the following pages, and the original nucleus of the colony (a). This mode of designation leaves open the use of other letters for mutants obtained in other ways. Only the (a) and (b) types will be considered in this paper.

Although morphological differences between (a) and (b) forms have not been observed, a definite distinction exists due to the presence or absence of an optically distinct capsule associated with a viscid condition of the growth in solid and in fluid media. The capsule was found only on (a) forms. Focusing on the periphery of the hanging drop of a diluted agar or a bouillon culture, one can frequently see the capsule, or a distinct spacing of the rods as they crowd together in the border of the drop. This spacing disappears as the marginal mass of bacteria begins to dry. The viscidity may be roughly estimated by the length of the thread which can be lifted up from agar or bouillon cultures after 1 or 2 days. The loss of viscidity and capsule occurred in all (b) forms studied. The presence of capsular substance was signalized by a heavy, glistening growth layer on sloped agar. This in some strains led to a slow sliding down of the growth from colonies on the slope, producing broad, vertical streaks. Such appearances may be seen in cultures of *B. lactis aerogenes* and some strains of the Friedländer bacillus. In (b) types, the growth was thinner, partly translucent, and easily distinguished on sloped agar from the (a) types. It did not form descending streaks of growth from colonies.

In bouillon after 1 or 2 days, (a) forms produced degrees of viscidity, evident, if not by the formation of cobweb-like threads at the end of platinum loop or wire, at least by the tardiness with which air bubbles rose to the surface when the fluid was shaken. These features were absent in (b) cultures. Fermentation reactions were not changed by the mutation, nor was there loss or gain of motility.

Immunological Characters.—Marked differences in the relation of (a) and (b) forms towards animal hosts were in evidence in all strains

studied. (a) forms are agglutinated only in very low dilution in the sera of animals treated with them or else not at all. This difference in agglutinability was first observed in rabbits immunized with killed cultures. Later rabbits were treated with both (a) and (b) types. In one experiment (a) serum agglutinated the (a) form in a dilution of 1:40, the (b) form in a dilution of 1:40,960. A rabbit treated with the (b) form produced a serum which agglutinated the homologous form at 1:40,960, but did not influence the (a) form at all.

To obtain sera from larger animals, the cow, being the normal host of the types of *B. coli* under investigation, was chosen. The first cow (1109) was treated with living 24 hour bouillon cultures of *B. coli* 1127a over a period of about 5 months. The injection was chiefly intravenous, the initial dose about 1 cc. The reaction was always fairly severe (1) so that the increase in dosage was very gradual. The final dose was 17 cc. Agglutination of the (a) form was barely indicated at 1:20, whereas the limit of agglutination of the (b) form was 1:1,280. A second cow was treated in a similar way with living cultures of 1192a. After 6 months of treatment with weekly injections the dose safely endured was 10 cc. Agglutination tests with this serum were negative towards the homologous culture at a dilution of 1:10. Towards the (b) form, however, clumping was nearly complete at 1:640, the highest dilution tried. The (b) form was clumped in high dilution by the immune cow serum of Strain 1127a and this serum agglutinated the (b) form of 1192. Serum of a normal horse caused complete clumping at 1:640, the highest dilution tried. Serum of a normal cow clumped completely at 1:80 (b) strains of both 1192 and 1127. Serum of a normal rabbit, however, failed to clump at 1:10 Serum of a normal calf (1242) showed slight clumping of (b) in a 1:40 dilution. The mutation process had so changed the (a) form that it became agglutinable in high dilutions not only in homologous immune serum but also in sera of untreated cow and horse. Cross-agglutination with (b) forms of *B. coli* strains showing wide differences in virulence towards guinea pigs was observed. The agglutinins in horse and cow blood may have been due to the accumulation of antibodies resulting from intestinal multiplication of various types of *B. coli*.

To determine the relation of leucocytes to the (a) and (b) forms the

blood of young calves was tested. The Wright technique was used and the capillary pipette containing the mixture of blood and bacteria incubated for 10 minutes at 37°C. The phagocytic index was depressed by the use of citrate, and was highest when defibrinated blood was substituted. Although the results were irregular there was no exception to the findings that the calf polymorphs were nearly inactive in the presence of the (a) type, but the (b) type was readily taken up. The presence of agglutinins in high dilution in the immune rabbit serum towards (b) organisms interfered with its use in the phagocytic test.

Virulence of (a) and (b) Forms.—Early in 1925 a study of the virulence of living cultures of various strains of *B. coli* from scours towards

TABLE I.

Virulence of the Original and the Mutant Form Compared.

Strain of *B. coli*	Minimum fatal dose		$\frac{(b)}{(a)}$
	cc.	*cc.*	
	(a)	(b)	
1085	0.04	1.0	25.0
1127	0.2	0.7	3.5
1192	0.04	1.0	25.0
223	0.06	0.7	11.6

guinea pigs was begun. The intraperitoneal route was chosen since subcutaneous injections failed to furnish any measurable data. The injection of the minimum fatal dose causes death within 24 hours. Very rarely an animal dies during the 2nd day. The reaction thus simulates the effect of a soluble or exotoxin. A comparative study of the minimum fatal dose of 24 hour bouillon cultures of (a) and (b) types yielded results shown in Table I. The last column expresses the relation between the minimum fatal doses of (a) and (b) forms. It will be noted that in these strains the minimum fatal doses of the (b) forms differ but little from one another, whereas the (a) forms show a greater spread.

Virulence in (a) types remained relatively constant when growths on sloped agar were transferred once in 4 to 6 weeks, incubated over-

night, and placed at 38–40°F. One strain (223) kept for 8 years in the manner described was still highly virulent for guinea pigs. In harmony with this persistence of virulence mutants did not appear in stock cultures kept in the manner described even after years of cultivation as repeated platings have shown.

The toxin production of (a) and (b) types was tested on calves as described (1). Two strains were used, Nos. 1069 and 1192. Given the same dose intravenously the characteristic respiratory symptoms were produced with equal severity by (a) and (b) filtrates of these two strains.

The uniform tendency of *B. coli* during mutation to lose certain characters indicating virulence and to acquire others also indicating loss of virulence marks this process as one of degeneracy but with reference to parasitism only. The degraded form persisted as such in cultures indefinitely. Two attempts were made to either restore or at least to modify it. One strain (1192b) was passed through a series of nine guinea pigs. Some of these died, others were chloroformed in 48 hours. Cultures were made from the peritoneal cavity and injected into the next of the series when 24 hours old. No increase in virulence or reappearance of viscid or capsular state was observed at the end of the series. A second attempt was made to change the mutant by passing it twice a day through bouillon tubes until more than 50 transfers had been made. At the end no changes towards the (a) form were manifest (2). The possibility of bringing the mutant back is not exhausted by these procedures. Not until such strains have been passed through the digestive tract of calves where the increased virulence of the (a) form may have been developed originally may we regard the change as irreversible.

DISCUSSION.

An examination of the voluminous literature on *B. coli* brings out certain data anticipating those briefly described. Baerthlein (3) in his extensive study of mutations of various species of bacteria mentions one group of paratyphoid strains, including two *suipestifer* and one *psittacosis* strain, which mutated on agar by sending out after several days a thinner, more translucent zone. In most writings the actual development of mutants from the original colony was not seen.

We are left to infer that cultures kept under certain conditions for a given period when plated presented two and sometimes more colony types. It is highly probable that the mutational changes went on in cultures kept in room temperature, exposed to diffuse light, and slowly drying out, although this is not stated. Massini (4) who was the first to observe mutations obtained his mutants from colony outgrowths resembling knobs (*Knöpfe*). No reference was found to the loss of capsules, or the disappearance of a viscid product in mutant forms.

Gratia (5) isolated ten mutants from a single original stock of *B. coli*. These differed from one another in motility and mucoid growth. The mucoid condition in his strain was a mutational state and not a property of the original strain as in our *B. coli* types. Changes in morphology for the cholera vibrio, typhoid, paratyphoid, dysentery, and colon bacilli are described by Baerthlein (3). One form appeared plump, the other slender.

Modification of agglutinability due to mutation was observed by Gratia in his colon strain and by Baerthlein in *B. enteriditis*. His (b) forms were agglutinated more easily than (a) forms. The (b) agglutinins were absorbed with great difficulty, if at all. In a *B. coli* mutant, the agglutination presented much the same peculiar conditions as we have described them for (a) and (b) forms. According to Baerthlein no changes in agglutinability were noted in the cholera vibrio and in typhoid and dysentery bacilli. De Kruif (6) noted changes in acid agglutination but not when immune serum was used (7) in his studies on dissociation in the rabbit septicemia organism. Reimann (8), in studies of pneumococci, finds the S or original form as type-specific, the R or mutant form as group-specific. The former is agglutinable only in its type serum; the latter in its own and in S serum. The distinction of type and group agglutinin (Reimann) was possibly foreshadowed by Burk (9) who found *B. coli* races from the human intestine agglutinated by sera from a variety of normal animals including rabbits. Mutation as interpreted by Baerthlein is more or less temporary and forms may pass back from mutant to original type. He does not touch upon virulence. Later studies by others indicate that mutation, in pathogenic forms at least, signifies a degradation with reference to virulence or parasitism. This view is taken by De Kruif

and Reimann and is clearly indicated in our results with *B. coli*. Recovery of the original level of virulence did not occur in cultures and not in passages through animals. As stated above a reversion to the (a) type in the original host animals is not negatived by experiments made thus far. Concerning changes in the character or concentration of toxins no statements have been found.

This brief and incomplete review suggests that the term mutation has been used to designate a variety of changes which bacteria may undergo during artificial cultivation. Some are obviously degradations and involve permanent loss of certain functions. Some are probably exaggerations or depressions of functions which can be brought back by certain procedures, as for instance the change back from R to S type by rapid transfers in bouillon (2).

The task of the future will be to redefine and classify the changes or mutations and evaluate them from different points of view. Especially desirable is a renewed study of those mutations in which definite functions are acquired, such as motility, fermentation of lactose (4), and of saccharose (10). These changes may be regarded as depressions or exaggerations or inhibitions rather than as new acquisitions until more exacting methods have been applied in a study of them.

GENERAL CONCLUSIONS.

On agar plates certain strains of *B. coli* from the ileum of calves suffering from diarrhea or scours promptly mutate and give rise to forms which have lost capsular substance, whose virulence has been greatly reduced, and which have gained very greatly in agglutinability and in being taken up by leucocytes. The original characters are not regained in cultures kept in the cold after development, nor in rapid transfers in bouillon, nor in passages through the peritoneal cavity of guinea pigs. Filtrates of 48 hour bouillon cultures contain as much toxin in the (b) as in the (a) form indicating no loss in this function.

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EXPLANATION OF PLATES.

PLATE 6.

FIG. 1. Agar plate of *B. coli* 1127a. After several days incubation the outgrowths were nearly of maximum size and continued to expand but slightly in room temperature under a darkened bell glass. The plate was photographed after 21 days.

FIG. 2. Agar plate of *B. coli* 1085a. Details as for Fig. 1.

PLATE 7.

FIG. 3. Agar plate of *B. coli* 1192a. Details as for Fig. 1.

FIG. 4. Agar plate of *B. coli* 223. On this plate a different form of outgrowth is illustrated. The mutant appears as a web-like expansion between clefts formed in the original colony.

All four plate cultures were prepared and photographed at the same time.

FIG. 1.

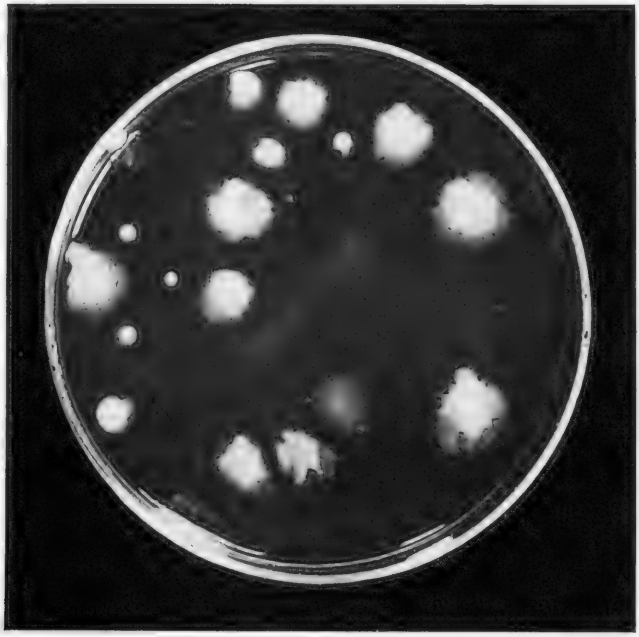

FIG. 2.

(Smith and Bryant: Pathogenic *B. coli* from bovine sources. II.)

FIG. 3.

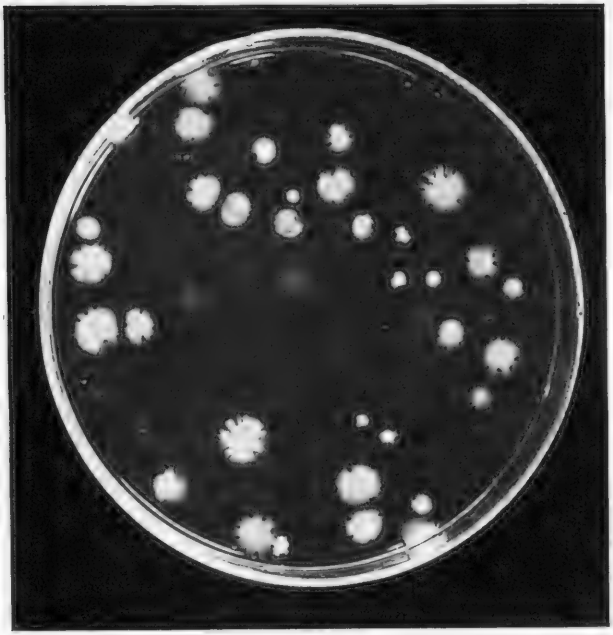

FIG. 4.

(Smith and Bryant: Pathogenic *B. coli* from bovine sources. II.)

STUDIES ON PATHOGENIC B. COLI FROM BOVINE SOURCES.

III. Normal and Serologically Induced Resistance to B. coli and Its Mutant.

By THEOBALD SMITH, M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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In view of the formidable differences in serological behavior of the original and the dissociated or mutant forms of *B. coli* described as appearing within 48 hours on agar plates made directly from intestinal contents,[1,2] it became desirable to analyze the virulence of the original and the mutant strain with the use of immune sera. To do this it became necessary to use the guinea pig in place of the cow or calf. This animal has been used in the past by various workers beginning with the early studies of the cholera vibrio by R. Pfeiffer. The intraperitoneal injection was chosen since the subcutaneous route fails to elicit any measurable reaction. To determine the minimum fatal dose bouillon cultures just 24 hours old were used. In these the turbidity of different strains and of the same strain in different lots of bouillon was markedly uniform and any more elaborate measure of dosage of the bacteria was dispensed with. The plating of definite dilutions of 24 hour bouillon cultures of the strain used chiefly in the experiments to be reported showed that there were present in 1 cc. 900 million of *B. coli* 1192a and 1 billion of the mutant (b).

The disease in guinea pigs following the intraperitoneal injection of the highly virulent or (a) form[2] was characterized by a prompt offensive and defensive reaction leading to death within 24 hours, or else only quiescence for 12 to 18 hours, loss in weight, and recovery

[1] Smith, T., and Little, R. B., *J. Exp. Med.*, 1927, xlvi, 123.

[2] Smith, T., and Bryant, G., *J. Exp. Med.*, 1927, xlvi, 133.

within 2 or 3 days. Very rarely an animal died after 18 to 24 hours. The minimum lethal dose may be titrated with nearly the same accuracy as is possible with diphtheria toxin. The effect of non-lethal doses may be roughly gauged by loss in weight. In early deaths, occurring between 6 and 10 hours, there is a trace of bloody fluid in the peritoneal cavity and indications of hemolysis. Certain viscera lying against the dorsal wall, such as the uterine horns and dorsal wall of cecum, may be spotted with hemorrhagic areas. Bacteria coat the peritoneum in large numbers. In animals dying between 12 and 24 hours, there is less congestion and hemorrhage and instead a beginning filmy exudate on liver and spleen. Bacteria may be very numerous or absent in films. All gradations are found between these extremes.

With the appearance of exudates, the viscera become covered with a translucent film of cells, not noticed at first until cover-slips are laid on the peritoneum and drawn away. Polymorphs are the only cell form at first and they increase in numbers with prolongation of life. When death occurs, in about 24 hours, there may be $\frac{1}{2}$ to 1 cc. of a ropy, opalescent fluid made up of leucocytes and bacteria.

In surviving guinea pigs, macrophages appear near the end of the first 24 hours and gradually increase in numbers so as to represent from one-fifth to one-half the number of cells present. Bacteria are very scarce at this stage. The cell exudate may be traced for 3 or 4 days, after which period it disappears. Occasionally whitish, viscid masses, 2 to 3 mm. in diameter, made up of polymorphs and macrophages remain in the folds of the omentum and spaces between the viscera. The lungs, at first normal in appearance, become greatly congested if death occurs as late as 18 to 24 hours. Usually from $\frac{1}{2}$ to 2 cc. of clear fluid is present in the pleural cavity at this time. Recovered guinea pigs, killed in 2 or more weeks, frequently have the spleen bound down by one or more firm adhesions to the abdominal wall.

The normal guinea pig weighing 350 to 400 gm. is capable of destroying a certain number of *B. coli* introduced into the peritoneal cavity. Among the strains isolated from calves the minimum fatal dose varied from 0.02 cc. to 0.5 cc. of a 24 hour bouillon culture. As a rule, three-fourths of this dose was still overcome by the guinea

pig. There was, however, a considerable fall in weight—from 40 to 50 gm.—when doses near the limit were injected, with a recovery of the original weight in 7 to 10 days. The injection of living *B. coli* thus gives almost as sharp a death point as is obtainable with diphtheria toxin. Of those that die, death occurs within 24 hours in fully 95 per cent. The bulk of the bacteria injected disappear from films within 24 hours, even in the rare cases that die after this time. In cultures from such animals made with loops rubbed over the viscera or dipped into traces of fluid present, usually a few colonies appear. In guinea pigs killed 2 or 3 days after inoculation, bacteria may frequently be cultured from the small clumps of cellular exudate filling interstices between viscera. After 4 or 5 days, these also are sterile.

Phagocytosis by polymorphs is evident between 12 to 24 hours, even in fatal cases, and increases with time. It is more pronounced following the injection of less virulent races. By the time macrophages appear, bacteria are scarce or absent. These cells are seen containing bacteria in very rare instances. In view of the usual function of macrophages in this experimental disease—ingesting polymorphs—the contained bacteria are probably within polymorphs in the macrophages. This is indicated when neutral red is used as a vital stain since the latter brings out the polymorph inclusions.

When a fraction of the fatal dose of a (b) mutant[2] of low virulence is given, the process followed by killing the animals at intervals indicates a rapid destruction of bacteria. When one-fourth the fatal dose is injected, representing about 0.25 cc. of culture fluid and equivalent to perhaps six times the number of bacteria in the fatal dose of the original or (a) strain, the number of living bacteria in the abdominal exudate falls to one-third within an hour. A small number may survive for 2 or 3 days in the lumps of polymorphs attached to omentum or viscera. The movement of polymorphs into the peritoneal cavity is more rapid in the presence of mutants and within 4 hours the peritoneal fluid is clouded with clumps of such cells. The cellular reaction beyond being more prompt does not differ in its further behavior from that already described for the (a) strain.

The toxicity, which maintains itself quite uniform in the same lot

of bouillon, must be retested when new lots of bouillon are used. If meat (veal) from young animals of nearly the same age is used the change from lot to lot is slight. If, however, meat from yearlings or older cows is used, the result may be quite different. In the case of Strain 1192a, the minimum fatal dose fell from 0.05 cc. to 0.02 cc. when meat from an older animal was used. Table I gives the results of one of several tests between the latter and a fresh lot of veal bouillon.

The Action of Immune Sera in the Protection of Guinea Pigs.—To study the factors involved in the varying behavior of *B. coli* in the peritoneal cavity of the guinea pig, immune sera were introduced into the problem. To obtain a fairly active serum cows were immunized. In what follows the sera of three cows were used. A

TABLE I.

Toxicity of Cultures Made from Different Lots of Meat.

No. of guinea pig	Weight	Dose (*B. coli*)	No. of bouillon	Results
	gm.	*cc.*		
1	375	0.02	2252b (veal)	Lives
2	380	0.024	2252b "	"
3	375	0.02	2239 (beef)	Dead in 11 hrs.
4	380	0.024	2239 "	" " 11 "

brief statement of two of these will be found in an earlier publication.[2] No. 1109 was treated intravenously with living 24 hour bouillon cultures of *B. coli* 1127a. No. 1231 received intravenous doses of *B. coli* 1192a, and No. 1232, only Berkefeld filtrates of 48 hour bouillon cultures of 1192a. A more complete history of these cows will be given in another publication.

The sera thus produced were tested on guinea pigs.[3] The minimum fatal dose of a 24 hour bouillon culture of 1127a injected into the peritoneal cavity of a guinea pig weighing 350 to 400 gm. was 0.2 cc. Of the homologous immune serum, 0.1 cc. mixed with the culture was the minimum life-saving dose.

[3] The method here employed of injecting a mixture of culture and serum into the peritoneal cavity of the guinea pig had been used by Grosso in testing scours sera. See Grosso, G., *Z. Infektionskrankh. Haustiere*, 1912, xii, 54.

The marked protective action of colostrum and of cow serum administered to new-born calves suggested the idea that normal cow serum might in itself neutralize the fatal effect of *B. coli* in the guinea pig. In Table II is shown a comparative test with specific immune serum (*B. coli* 1127a) and normal cow serum. The immune serum protected against the minimum fatal dose in 0.1 cc. but not in 0.05 cc. amounts. The normal serum failed to protect in 1 cc. doses and a later test with 1.5 and 2 cc. did not retard death. Similar tests with sera from four other cows in doses of 1 to 2 cc. failed to

TABLE II.

Effects of Normal Serum (Cow 1100) and Immune Serum (Cow 1109).

Weight of guinea pig	24 hr. bouillon culture	Serum	Source of serum	Remarks
gm.	*cc.*	*cc.*		
450	0.2	—	—	Dead in 11 hrs.
460	0.2	0.1	Cow 1100	" " 12 "
465	0.2	0.2	" 1100	" " 11 "
440	0.2	0.1	" 1109	Lives
430	0.2	0.2	" 1109	"
525	0.2	0.05	" 1109	Dead in 12 hrs.
550	0.2	0.1	" 1109	Lives
510	0.2	0.2	" 1109	"
410	0.2	0.5	" 1100	Dead in 12 hrs.
425	0.2	0.7	" 1100	" " 8 "
440	0.2	1.0	" 1100	" " 12 "
490	0.2	—	—	" " 12 "

retard death. In these different tests, the sera had been stored for some time and any toxic effect of the 2 cc. doses was not noticed.

Strain 1192a was fatal to guinea pigs of the same weight in doses of 0.05 cc. In a preliminary test the serum of the cow treated with living bacteria saved life in 0.5 cc. doses. The "filtrate" serum failed to do this in the same dose. Later tests demonstrated that 0.05 cc. of the living-culture serum protected against 0.06 cc. culture, a trifle more than the minimum fatal dose. Table III illustrates the action of the immune serum at this stage.

A continuation of the treatment of both cows resulted in stronger immune sera. That of the animal treated with intravenous doses of

living cultures 11 months protected guinea pigs in doses of 0.005 cc. against $1\frac{1}{4}$ times the surely fatal dose. The serum of the cow treated with filtrates failed to do this in doses less than 0.5 cc. The law of multiples effective with diphtheria toxin and antitoxin mixtures fails when applied to the pathogenic action of living cultures of *B. coli*. In several trials, after the minimum serum dose effective in neutralizing $1\frac{1}{4}$ times the minimum fatal dose of culture fluid had been established as 0.005 cc. serum protecting against 0.025 cc. culture, a dose of 0.2 cc. serum, or 40 times the above dose, protected against

TABLE III.

Effects of Immune Cow Sera 1231 (Living Culture) and 1232 (48 Hour Culture Filtrate), Strain 1192a.

Weight of guinea pig	Dose of culture	Dose of serum	Result
gm.	*cc.*	*cc.*	
350	0.04	—	Lives
355	0.05	—	Dead in 15 hrs.
370	0.06	—	" " 10 "
350	0.05	0.5, No. 1231	Lives
355	0.06	0.5, " 1231	"
370	0.075	0.5, " 1231	"
340	0.06	—	Dead in 5 hrs.
340	0.06	0.2, No. 1231	Lives
340	0.06	0.3, " 1231	"
350	0.06	0.1, " 1231	"
375	0.06	0.05, " 1231	"
350	0.06	0.5, " 1232	Dead in 8 hrs.
340	0.06	1.0, " 1232	" " 8 "

0.1 cc. culture, or 4 times the fatal dose. 0.1 cc. serum did not protect. Assuming in the latter case the protective dose to be actually 0.15 cc. serum, 4 times the minimum fatal dose requires 30 times the minimum serum dose.[4]

Although the immune cow sera showed a very definite protective action on guinea pigs, they failed to neutralize the toxic action of

[4] If we regard 0.02 cc. culture as controlled by the natural resistance of the guinea pig and subtract this from the culture doses used, we still have a culture ratio of $\frac{0.08}{0.005}$, or 16, to a serum ratio of $\frac{0.15}{0.005}$, or 30.

filtrates on calves. The sera were tested on fifteen calves. The dose of filtrate was 2 cc., that of the sera from 2 to 6 cc.

The respiratory symptoms already described[1] as produced by 2 day filtrates of both (a) and (b) forms of *B. coli*, were not neutralized or modified to any extent when mixed with either "living culture" or "filtrate" serum before injection. Thus the dose of filtrate usually injected, when mixed with 2 cc. and 3 cc. homologous "living" serum (1127a) failed to limit appreciably the violent reaction. The

TABLE IV.

Effect of Cow Serum (B. coli 1192a) on Pathogenic Action of Four Other Strains.

Weight of guinea pig	Strain	Culture dose	Serum dose	Result
gm.		*cc.*	*cc.*	
365	223a	0.07	—	Dead in 14–16 hrs.
360	223a	0.06	—	" " 24 "
365	223a	0.07	0.5	Lives
360	1085a	0.04	—	"
385	1085a	0.05	—	Dead in 14–16 hrs.
365	1085a	0.06	0.5	" " 29 "
355	1092	0.08	—	" " 29 "
360	1092	0.1	—	" " 14–16 "
360	1092	0.1	0.5	" " 22 "
350	1228	0.05	—	Lives
360	1228	0.06	—	Dead in 40 hrs.
360	1228	0.07	0.5	Lives

same was true of Sera 1192a, prepared with living cultures and filtrate respectively. Thus 2 cc. filtrate of (a) type or (b) type mixed with 2 cc. undiluted, homologous immune cows' sera both "living" and "filtrate," failed to moderate the reaction. In one calf (1159) the addition of 6 cc. "filtrate" serum did not check a severe reaction. The lungs of this calf 2 days after injection were still focally hemorrhagic and generally congested. If any mitigation of the toxic effects is to be produced by immune sera, it obviously will require prolonged immunization and large doses of the immune sera.

Polyvalency of Immune Sera.—The serological relationship of the

different strains of *B. coli* isolated from the ileum during scours or in normal condition was determined by using immune cow serum. When the minimum fatal dose was mixed with the serum a certain grouping was made possible. In Table IV two strains were obviously modified in their pathogenic action by the serum. In two others the same quantity was not sufficient to prevent death.

The overlapping of the protective action of sera from two cows treated with different strains is brought out in Table V. Where the

TABLE V.

Polyvalency of Immune Cow Serum.

Strain	Minimum fatal dose	Protective dose of serum in 0.5 cc. or less		Culture character
		1127a	1192a	
	cc.	*cc.*	*cc.*	
223a	0.06–0.07	>0.5	0.5 or less	Saccharose + (motile)
1069a	0.5	>0.5	—	
1085a	0.04	0.2–0.3	>0.5	
1085b	1.0	0.5	—	
1092	0.1	0.3–0.4	>0.5	Saccharose + (motile)
1127a	0.2	0.1	—	
1127b	0.7	0.03	—	
1179	0.4	>1.5	—	
1189	0.5	>1.5	—	
1192a	0.04	0.3	0.05	
1192b	1.0	—	0.1	
1197	0.5	0.5	—	
1208	0.15	0.3	—	
1228	0.05	0.5	0.5 or less	Saccharose +
1358a	0.08	—	0.5 " "	

amount of serum needed is indicated as higher than the doses tried, there is of course the possibility that the serum has no effect whatever. Thus Strain 1179 probably belongs to an entirely different serological group. There is also the possibility that by prolonged treatment of the cow the serum might become protective in the doses used.

It is of interest to consider the relative neutralizing power of immune serum towards the (a) and (b) forms. In an early test of serum of Cow 1109 (*B. coli* 1127a), 0.1 cc. serum inhibited the surely

fatal dose of 0.2 cc. of the homologous strain. 0.03 cc. of the same serum inhibited the surely fatal dose of 0.7 cc. of the (b) mutant. A more detailed test was made with an immune serum of Cow 1231 (*B. coli* 1192a). The final tests are given in Table VI. Tests made with the same drawing of serum on the (a) form showed that the surely fatal dose of 0.04 cc. ($1\frac{1}{3}$ × M.F.D.) was inhibited by 0.005 cc. serum.

There is thus very little difference in the protective power of the (a) serum on the surely fatal doses, 0.04 and 0.6 cc., of the (a) and (b) forms respectively.

Non-Specific or Natural Resistance in Guinea Pigs.—It has been stated that guinea pigs are capable of disposing of considerable num-

TABLE VI.

Immune Cow Serum 1192a and Culture B. coli 1192b.

Date of test	Weight of guinea pig	Dose of culture	Dose of serum	Result
	gm.	*cc.*	*cc.*	
Feb. 4	370	0.5	—	Dead in 12 hrs.
" 4	360	0.6	—	" " 12 "
" 9	350	0.6	—	" " 15–16 "
" 9	355	0.6	0.005	Lives
" 9	330	0.6	0.02	"
" 11	345	0.6	—	Dead in 13 hrs.
" 11	355	0.6	0.0025	Lives

bers of colon bacteria even when of maximum virulence. This phenomenon may be ascribed to a natural immunity. It had been shown by R. Pfeiffer and Issaeff[5] that by preparing the abdominal cavity of guinea pigs by the injection of bouillon, salt solution, and the like, an increased resistance towards the cholera vibrio could be produced. The same is true when *B. coli* is used. When guinea pigs receive into the peritoneal cavity 2 cc. bouillon, the minimum fatal dose of *B. coli*, or even a larger dose, is readily borne when injected 2 days later. If the experiment is continued and the same dose injected 10 to 14 days later, the guinea pig may die. In other words, the animal has lost in resistance during this period even though the

[5] Pfeiffer, R., and Issaeff, *Z. Hyg. u. Infectionskrankh.*, 1894, xvii, 355.

loss in weight following the injection of the first fatal dose has been amply compensated. Repeated trials were made to eliminate any possible errors in the tests. All showed that at least a certain number of guinea pigs had not recovered their normal resistance. In Table VII a final test is given. Eight guinea pigs received 2 cc. bouillon into the peritoneal cavity. 2 days later their weights had all increased. Three of them received 0.02 cc., and three, 0.025 cc. culture fluid. 2 days previous 0.022 cc. was found fatal. 11 days later all eight received doses from 0.016 cc. to 0.022 cc. in amount. Of these, two having received 0.02 cc. died within 10 hours. It will

TABLE VII.

Original weight of guinea pig	All injected intraperitoneally, 2 cc. of sterile bouillon				Result	Weight 9 days after 2nd inoculation
	Injected 2 days later		Injected 11 days later			
	Weight	Dose of *B. coli* culture	Weight	Dose of *B. coli* culture.		
gm.	*gm.*	*cc.*	*gm.*	*cc.*		*gm.*
385	—	—	440	0.02	Lives	400
355	—	—	450	0.022	"	430
375	395	0.02	420	0.016	"	415
370	400	0.02	430	0.02	Dead in 10 hrs.	—
375	390	0.02	430	0.018	Lives	445
370	400	0.025	430	0.016	"	430
365	385	0.025	445	0.02	"	440
390	400	0.025	460	0.02	Dead in 10 hrs.	—

be noticed that 0.022 cc. did not kill a control; and that of the two that died, one had easily borne a dose one-fourth higher than the one which proved fatal. The other died following the same dose originally borne successfully. In this experiment all bouillon used for *B. coli* cultures was from the same lot.

Following an initial dose of *B. coli*, the recovered animals in a few instances have the spleen partly bound down by adhesions. The peritoneum recovers fully its original normal appearance. Permanent opacities and infiltrations are absent. The failure to resist the second dose does not appear to reside in any distinct anatomical defects or changes.

DISCUSSION.

In attempting to formulate the mechanisms by which the guinea pig is injured as well as protected we have several phases of the work to assist us. The study of the effect of the filtrate or toxin on calves shows us that most, if not all, of the pathological effect of the bacillus may be accounted for by its toxic filtrate. The extreme dilatation of the capillary system followed by hemorrhage as found in calves described also its action on guinea pigs, in spite of the fact that this species appears to be relatively indifferent to the culture filtrates. The guinea pigs either die within 24 hours or appear lively after 12 hours. Very few die on the 2nd day. The offensive weapon, then, of *B. coli* appears to be its soluble, diffusible toxins. Another fact which favors the view of the toxin action of *B. coli* is the difference in virulence due to different lots of bouillon. With *B. coli* 1192a the minimum fatal dose increased 2 to $2\frac{1}{2}$ times from one lot to another.

The offensive action of the guinea pig probably is not a neutralization of this toxic filtrate, for in calves we find that a relatively large amount of immune serum mixed with filtrate does not relieve the symptoms due to the toxin. The guinea pig probably protects itself by some stored natural protective substance which, if present in sufficient quantity, checks the bacteria. If not, the bacteria continue to multiply and toxin production goes on until death ensues. This substance is mobilized by the injection of non-specific substances such as bouillon, so that a minimum fatal dose is easily borne. On the other hand, it appears to be used up by the injection of cultures and not fully restored even after 10 to 14 days, because at this time a second fatal dose or even a smaller dose may be fatal. If specific immunity consists in an overproduction of this substance it follows that resistance can be induced in guinea pigs only very gradually. It is highly probable that this substance destroys the bacteria by lysis, as is known for the cholera vibrio. The cooperation of polymorphs in this phase is highly doubtful. The cells appear too late on the scene. The macrophages come still later, after the fate of the guinea pig has been settled.

To protect itself against this factor in the host, certain colon bacilli have developed a capsular substance. That this bacterial substance is primarily protective is suggested by its absence in mutants. They

retain the toxin-producing capacity, but lose, largely or wholly, the capsular, viscid substance and with it their specific resistance to agglutination and phagocytosis. Their virulence towards the guinea pig dropped in one culture to $\frac{1}{3}$, in several others to $\frac{1}{25}$ of the original dose. This change is completed a few days after isolation on agar plates. That death is still caused by the mutants is accounted for by the still normal toxin production. The large minimum fatal dose of living bacilli of mutant type is necessary since this type is readily destroyed and must be present in excess at the start.

The injection of living cultures of *B. coli* of the (a) or viscid type

TABLE VIII.

	Bacterial activities	Host activities
B. coli (a) type (M.F.D.: 0.04 cc. or 0.025)	Toxin ——→ kills (offensive)	(Normal) antibody towards toxin, if any, increased very slowly or not at all by treatment
	Capsular substance (defensive)	←—— (Normal) antibody greatly augmented by treatment (vaccination, immunization)
B. coli (b) type (M.F.D.: 1.0 cc. or 0.6)	Toxin ——→ kills (offensive)	←—— Unless host overpowered by toxin at the start, bacteria killed readily by antibodies
	Capsular substance (present in traces or absent) (defensive)	

leads to the production of an immune serum which mixed with cultures and injected has a definite and potent protective action whereas normal cow serum has none in evidence in the technique used. When such normal serum is injected in quantities up to 2 cc., with cultures, death is not retarded. As stated above, the immune serum is not antitoxic. It probably cooperates with the normal forces of the guinea pig in suppressing multiplication and destroying the bacteria, since only a few survive in the peritoneal cavity after 24 hours. The interrelations of the several host-parasite activities, as suggested by the data given, are presented in Table VIII.

The presence of normal, antibacterial forces in the body and the production of highly specific antibodies by the repeated injection of

cultures have always been more or less incompatible factors in theories of immunity. The results reported in these papers offer the following hypothesis towards harmonizing these factors. The peritoneal cavity of the guinea pig and its walls (endothelium?) contain some substances normally which destroy the injected bacteria either by removing a protecting, viscid envelope and so exposing the organism to lysis, or by checking multiplication in one or more ways such as making the bacterial membrane more permeable to lytic factors or less permeable to nutritive elements. These substances are mobilized by the preliminary injection of bouillon. When some animal host is repeatedly treated with cultures, some one of these protective factors is highly developed in accordance with the capacities of the host. This gives the immune serum a one-sided, more or less specific effect, since the bacteria are suppressed in a certain way only.

According to this hypothesis, each host might furnish a serum of slightly different antibacterial activity towards the same microorganism according to the specific bacterial factor most easily handled by such host. It is conceivable that if the toxin of *B. coli* is antigenic, some host may protect itself chiefly by developing an antitoxin. The reason for serum specificity towards different biological groups of bacteria may be explained in a similar way, in that the bacteria themselves may present several widely different points of attack. Natural immunity according to this theory is simply a reservoir of numerous natural possibilities to be stimulated according to the different patterns presented by the microbes and the different capacities of the hosts for overdeveloping the individual contents of this reservoir. In particular the outer functional element, the ectoplasm, capsule, membrane, of the parasite, whatever it may be called, seems to stand out as the most important of the bacterial factors to be met by the host. If there is an active secretory function throwing off protective material, this, becoming diffused through the system of the host, may bring many cell territories to cooperate in increasing the output of protective antibodies. Immunity may come quickly and permanently, or else the microorganism gains the upper hand equally rapidly as in anthrax. The less the outer functional element is concerned in secreting and excreting protective substances, and the more it is concerned in transforming itself into a

locally protective envelope difficult to attack, the more the immunizing process becomes localized, and the slower the multiplication of the bacteria and the more difficult the acquisition of general immunity becomes.

The paratyphoid group, probably an offshoot of the colon group, may serve to illustrate this hypothesis. The paratyphoid bacillus produces a toxin which has similar effects on calves. It has no original capsular or viscid substance. None of the group yields any satisfactory antisera such as are readily produced by the frequently capsulated bipolar pneumococcus and colon groups. These invasive forms are however able to reach sensitive tissues and to produce their toxin in the close vicinity of highly sensitive cells. What this may mean is illustrated by *B. coli*. The toxins of this group introduced into the subcutis have little systemic effect. Brought in contact with the pulmonary epithelium artificially, as in intravenous injections, a very small amount produces profound effects.

CONCLUSION.

The interrelations between bacterial toxins, bacterial capsular (mucoid, viscid) substance, and certain normal protective factors in the guinea pig are studied with the aid of bacterial mutants and immune serum, and the results formulated in an hypothesis.

STUDIES ON PATHOGENIC B. COLI FROM BOVINE SOURCES.

IV. A Biochemical Study of the Capsular Substance.

By DOROTHEA E. SMITH, Ph.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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The carbohydrate gums which have been obtained from bacteria in sufficient quantity for chemical study are few in number. The early workers were concerned with capsular material as such, and the possibility of its precipitation by homologous immune serum had not been thought of. A bibliography and review of the literature are to be found in the papers of Heidelberger and Avery (1), or of Heidelberger (2).

Preisz (3) studied in detail the rôle of capsule and capsular material in infectious disease. He made preparations of capsular material in considerable amount. He believed it to be a mucin and stated that it gave no reducing sugar reaction. Whether the substance was prepared from virulent or avirulent strains, it removed from sera the factors responsible for lysis or the killing of anthrax bacilli (page 399). But he did not consider this a specific reaction, because similar material from cultures of organisms resembling *B. anthracis* also removed bactericidal substances from antianthrax sera *in vitro*. He thought that in the last stages of infection in the rabbit, capsular substance was present in blood and exudate in solution, neutralizing the antibody. He maintained that there was throughout a parallelism between virulence and the possession of a capsule.

With observations from studies such as this two lines of evidence converged towards and merged into the present concept of a polysaccharide reacting specifically with homologous serum, responsible for type specificity, but not eliciting antibody formation, and in all probability connected with the possession of a capsule and virulence in certain cases.

1. In 1921 Zinsser (4) called attention to the immunological importance of the non-protein constituents of bacterial cells. This frac-

tion which reacts with specific serum antibodies was termed "residue" in order not to imply too accurate an idea of its chemical structure. He believed this to represent the so called "haptene" of Landsteiner (5), for Landsteiner in his studies upon chemical modifications of precipitable antigens foretold the probable existence of materials which would react with antibodies without themselves being capable of inducing antibody formation when injected into the animal body. These conditions Zinsser's "residue" fulfilled, and the suggestion was made that they represented the haptophore group split off from the antigen as a whole and molecularly too small to induce antibody formation. It was also stated that this "residue" was analogous to the substances previously found by Cole (6) and by Dochez and Avery (7) in the blood and urine of typhoid and pneumonia patients.

2. The mass of evidence presented in the fundamental papers of Avery and Heidelberger (2) and Heidelberger and Avery (2) leaves us without grounds for doubting that it is a protein-free preparation that reacts specifically with homologous precipitating antibody and is responsible for type specificity. Very marked chemical differences were found correlated with serological specificity.

In the work of Mueller (8) and of Heidelberger, Goebel, and Avery (9) it was suggested that the "residue" or soluble specific substance is either identical with or connected with the capsule. But a question very naturally arises, why then is it possible to obtain "residue" from such apparently uncapsulated organisms as the tubercle bacillus, the staphylococcus, the meningococcus, and the influenza bacillus (10), as well as from the capsulated types of pneumococcus and the Friedländer bacillus? Is the capsule due to an increased production of a substance always present in the bacterial cell, a substance capable of reacting with antibody, but not of eliciting its formation on injection? Such a substance might represent the "haptophore" group of Ehrlich, and meet the conditions of the "haptene" prophesied by Landsteiner. Another question that naturally arises is the relation between capsular substance and virulence. A brief survey of the literature at once shows us that the possession of a capsule is almost universally regarded as increasing the virulence of an organism. This might be brought about in two ways. In the first place the capsular substance might act as a morphological factor, not dissolving away from

the organism, and insulating it from antibody action. Secondly, it might protect the organism by reacting in solution, at some distance from the bacterial cell, with antibody.

EXPERIMENTAL.

The possession of a number of mutating cultures of *B. coli* described by Theobald Smith and associates (11–13) offered an opportunity to look for the answers to the above questions in the case of this one species. It should be possible to find out if more precipitable carbohydrate is obtainable from the capsulated than from the non-capsulated strain from equal areas of culture surface, what its probable relation to the greater virulence of the capsulated strain may be, as well as something about its chemical properties.

A consideration of the literature leads us to expect that a capsule would increase the virulence of an organism. This is, therefore, a very unique opportunity to obtain a quantitative estimate of the extent to which it enhances virulence. We think we are justified in excluding other factors, because the organisms are so alike. One, called the (b) form, is derived as a mutant from a colony of the other, called (a). Precipitation in the culture filtrate of either one is caused by the serum of the other, as well as by its own serum, as will be shown later. Filtrates of bouillon cultures, 24 to 48 hours old, of both (a) and (b) forms when injected into calves exert marked toxicity (11), but guinea pigs are only slightly sensitive.

These cultures of *B. coli* also afford an opportunity to test out the suggestion that "residue" or soluble specific substance is either identical with, or connected with, the capsule; because, if a certain substance were found to be present in extracts of the capsulated bacillus, and either not present at all or present in very small amounts in similar extracts of the mutant, it would be good evidence that it was indeed the material of the capsule. Of course it does not prove that there are not small amounts of other substances present, such as mucin. In the case of an alkaline extract of an extremely viscid strain of Friedländer's bacillus previously studied by the writer, there was present a large amount of substance or substances containing very little nitrogen in addition to the specifically active carbohydrate.

But in the case of the capsule of the colon strain there was nothing similar, at least not in appreciable amount

In what follows, the special strain of *B. coli*, 1192a, and its mutant 1192b, studied by T. Smith (11–13) was used throughout. In preliminary experiments, in which an alkaline extract of the bacteria was centrifuged and the supernatant precipitated with 2 volumes of 95 per cent alcohol, the precipitate, in the case of the viscid strain, was found to show a white ring very promptly at a dilution of 1 part in 100,000 by weight, with homologous serum. The mutant, non-viscid strain yielded a much less active precipitate, which gave a similar test more slowly and only up to a dilution of 1:1000 by weight, with either serum. It would seem therefore that in one case there was about a hundred times as much precipitable substance as in the other, so it seemed worth while to try to isolate the substance.

Preparation of Soluble Specific or Capsular Substance.—In all about 90 ordinary tin pie plates, each covered with a tin plate of larger size, were poured and inoculated. The ordinary stock veal infusion agar kept on hand in the laboratory was used, with the addition of 0.1 per cent dextrose just before pouring. The agar surfaces were very liberally inoculated with a suspension from 24 hour agar slants in normal saline. After 2 days' incubation, the growth was removed in distilled water (14). In this condition it was still extremely viscous. The bacterial emulsion was diluted to a volume of 500 cc., treated with about 75 cc. of 10 per cent KOH, warmed to 70°, and kept at that temperature about 30 minutes. After neutralizing and making slightly alkaline to litmus, adding about 10 gm. of sodium acetate and 150 cc. of 95 per cent alcohol, a clear supernatant was obtained after centrifugation and the sediment of bacterial bodies rejected. The substance in the supernatant was precipitated on adding 95 per cent alcohol up to 1.2 volumes. It was partially purified by repeated solution and precipitation with 1.2 volumes of 95 per cent alcohol, added drop by drop with mechanical stirring, in the presence of plenty of electrolyte in the form of sodium acetate, usually from solution distinctly alkaline to litmus. Precipitation from acid solution was also employed. The discarded supernatant contained much orange-brown material and very little specific substance as determined by the precipitin test. Precipitation with alcohol was continued 5 to 7 times, usually until the supernatant was practically colorless. The final precipitate, which unlike similar Friedländer material dissolves readily in warm distilled water, was put through a Berkefeld filter, after sufficient dilution, if still turbid. After concentration to convenient volume and reprecipitation with alcohol, it was washed with alcohol in increasing concentration and dried *in vacuo* over sulfuric acid. The yield from 90 plates was somewhat over 2.3 gm. It was active with homologous serum when diluted to 1 part in 2 millions, using the Fornet-Müller ring test.

Description and Analysis.[1]—The biuret test was negative. There was no precipitate (in ½ per cent solutions) with tannic acid, phosphotungstic acid, Esbach's reagent, 5 per cent copper sulfate solution, 10 per cent uranyl acetate solution, or saturated barium hydroxide solution. There was no color with iodine. The Molisch test was strongly positive. But with 10 per cent basic lead acetate there was heavy precipitation and with 10 per cent ferric chloride a fairly heavy precipitate soluble in excess of the reagent.

The white, fluffy powder is readily soluble in hot water. In even a 1 per cent solution it is viscous and opalescent, somewhat resembling starch. It is not stringy, like the original growth on agar. It is very readily hydrolyzed by boiling with acid, and the hydrolysate reduces Fehling's solution, showing that a reducing sugar is present. It gives a slight naphthoresorcinol test for glucuronic acid and a slight test with orcinol, indicating the presence of glucuronic acid, but too faint for a pentose. There is a faint turbidity on boiling with barium hydroxide which is not removed by the addition of hydrochloric acid, thus indicating the presence of a hydrolyzable sulfuric acid. Very likely this is due to an impurity.

The substance, on drying in an Abderhalden dryer at 100°, lost 11.34 per cent of water. The elementary analysis, which was very kindly done by Dr. Elek, was as follows:

Ash 9.24 per cent —
C = 42.26 per cent
H = 5.82 " "
S = 0.6 " "
N = 0.6 " " (my own figure, micro-Kjeldahl, on 10 mg. portion)

Calculated on ash-free basis —
C = 46.56 per cent
H = 6.41 " "

For $(C_6H_{10}O_5)_x$ —
C = 44.4 per cent
H = 6.2 " "

So the carbohydrate nature of the substance is evident.

1 gm. of powder was used in the preparation of an osazone. It was hydrolyzed by boiling in 10 cc. of 2 per cent hydrochloric acid

[1] The writer was greatly assisted by Dr. P. A. Levene in this portion of the work.

under return condenser. The reaction was carefully followed by observing the rotation and the reducing sugar present in the solution from time to time.

Rotation		Reducing power equivalent to glucose
Initial	0.05	—
After ½ hr.	0.06	—
" 1½ hrs.	0.06	800 mg. (about 80 per cent)
" 5½ "	0.08	785 "

Since the values in the above table showed no significant change, heating was stopped, and the hydrolysis mixture neutralized with sodium hydroxide till only slightly acid to litmus. It was filtered and concentrated under reduced pressure to 60 cc. To obtain a phenylosazone it was warmed on the water bath with 2 gm. of free phenylhydrazine dissolved in glacial acetic acid. After 45 minutes on the water bath, it was heated a trifle more and immediately filtered through a folded filter. To this filtrate a second time phenylhydrazine in glacial acetic acid, to the amount of 1 gm., was added and the solution kept on the water bath as long as an osazone was forming. This was again filtered off, and a third precipitate of osazone obtained from the filtrate after standing about 1 hour more. The first lot of osazone was kept separate, but the second and third, which had a similar appearance, were combined. Each precipitate was taken up in methyl alcohol to remove adhering impurities, so that the final osazones were free from oil drops and consisted of long crystalline needles or rosettes.

The melting point of the first was 196°C. The second sintered at 180° and decomposed at 195°. Neither showed optical rotation. The second was analyzed for nitrogen, with the following results: From 0.0620 gm. substance was obtained 8.30 cc. nitrogen at 763.3 mm. Hg and 22°C. This is equivalent to 15.55 per cent nitrogen. (Calculated for $C_{18}H_{22}O_4N_4$, N = 15.63 per cent.)

The fact that the observed rotation of the hydrolysate was very low for the reducing power, and that the osazone seemed to be entirely inactive optically, makes it seem likely that the hydrolysis mixture contained at least two hexoses, one dextro- and the other levorotatory. The osazones were not separable, or they may have been identical. From these facts it seems established that the substance obtained

from the colon bacillus belongs in the class of carbohydrates, as shown by the elementary analysis, that it contains glucuronic acid, and hexoses. The total reducing power of the sugars obtained on hydrolysis calculated as glucose was equivalent to 80 per cent of the weight of the substance. The substance thus differs from all the others reported.

Attempt to Relate the Specific Carbohydrate Obtained from the Capsulated Bacillus to Its Greater Virulence.—The question naturally arose[2] as to whether it were possible to find out how the capsular material acts to increase virulence. Whether it may function as a morphological capsule, or partly dissolve and thus act at a distance by neutralizing the antibody in the blood of the host.

TABLE I.

Guinea pig No.	Weight	Culture	Extract	Result
	gm.	*cc.*	*mg.*	
1	355	0.3	—	Lives
2	350	0.6	—	"
3	350	0.9	—	"
4	360	1.2	—	Dead in 22 hrs.
5	355	0.3	10	Lives
6	350	0.6	10	"
7	350	0.9	10	Dead in 10 hrs.

It seems, from a consideration of the following observations, that in the case of the viscid strain the substance must be acting to a large extent as a closely adherent protecting layer, probably permitting multiplication of the organisms before they can be destroyed. We know from the papers by Smith and associates (11–13) on these mutants that capsulated strains are practically not phagocyted at all. If capsular substance is of significance in the production of virulence it may very likely be in connection with non-phagocytability.

A few experiments were made on guinea pigs to see if the substance extracted from cultures as a carbohydrate had any influence on the course of the disease produced by *B. coli*. The mutant (b) was tried

[2] For suggestions at this point the writer is particularly indebted to Dr. Hans Zinsser and Dr. J. H. Mueller.

first. Table I illustrates the procedure for testing any increase in virulence due to admixture of the living 24 hour bouillon culture with the extract. This was ground up in normal saline. The injection was made into the peritoneal cavity of guinea pigs.

An apparent increase in virulence may be deduced from the table. Several other tests yielded similar data. The (a) form was tried next to see whether it also might be favored by the preparation. 5 mg. of the preparation in normal saline were mixed with graded amounts of the culture and the mixture injected into the peritoneal cavity. The minimum fatal dose was definitely lowered. When, as controls, 5

TABLE II.

1192a and 1192b grown 24 hrs. in flasks containing 75 cc. of veal bouillon.

Serum of rabbit injected with strain	Capsulated strain, 1192a			Mutant, 1192b		
	Filtrate not diluted	Diluted 1/2	Diluted 1/4	Filtrate not diluted	Diluted 1/2	Diluted 1/4
(a)	+*	Tr.	0	+	+	Tr.
(b)	+	0 (?)	0	+	0 (?)	0
1192a and 1192b grown 48 hrs. in exactly similar conditions.						
(a)	+	?	0	+	+	Tr.
(b)	+	0	−	+	0	−
1192a and 1192b grown 3 days in exactly similar conditions.						
(a)	+	+	0	++	+	Tr.
(b)	+	+	0	++	+	0 (?)

* + means a ring that can be seen distinctly. None of these rings were heavy.

mg. of gum tragacanth, and a feebly turbid homogeneous suspension of aleuronat, probably less than 5 mg., were injected with *B. coli*, the same increase in virulence was observed.[3] This method of demonstrating the possible relation of the carbohydrate to virulence was not pursued any farther, since it was evident that some other method would have to be worked out, eliminating mere injury to the peritoneum. The protocols relating to the test with the (a) form are therefore omitted.

[3] See also Benians (15).

It is to be noted that the amount of precipitable substance found in filtrates of early cultures is actually very small, both in the (a) and the (b) forms. This is brought out in Table II.

The two rabbits yielding the highest titer serum, Nos. 1 and 2, had been given intraperitoneal injections, 5 days apart, of increasing amounts of killed growth from 24 hour agar slants emulsified in normal saline. It is impossible to clear the culture of the capsulated strain by centrifuging. An attempt was made to see if any rings could be distinguished in a dilution of 1:8 to eliminate turbidity, but there was not enough precipitable substance to give a reaction at this dilution. So recourse to filtration through a small Berkefeld was necessary. Saline was always passed through first and tested to insure absence of anything giving a ring with the most potent serum (a). Then half the 75 cc. of culture fluid was passed through and rejected, since precipitable substance might be adsorbed by the filter at first. Inasmuch as all the material of all preparations made had been put through Berkefeld V's or N's and there was no trouble with adsorption, the precaution of using only the last part of the filtrate would seem sufficient.

DISCUSSION.

The condition presented by *B. coli* (a) and (b) was quite different from that found by Dochez and Avery (7) when studying the pneumococcus. During the early stages of vigorous growth this organism forms a readily soluble substance which diffuses into the culture medium *in vitro*, and in human and animal infections is present in the blood and urine. The writers could not demonstrate that this substance was responsible for the intoxication occurring in lobar pneumonia, but they were able to state, after the study of 112 cases, that if large amounts were excreted, the outcome was usually fatal. The table on page 479 of Dochez and Avery's paper is particularly instructive. This is from the study of a flask inoculated with an organism of Type III, which forms somewhat more precipitable substance than I or II. After only 4 hours, the undiluted filtrate showed heavy flocculation with homologous serum. A 24 hour culture showed a heavy precipitate at a dilution of 1:30. A trace was recorded at a dilution of

1:120. Evidently the material diffuses out into the culture fluid in considerable amounts.

When we consider the capsulated *B. coli*, we find an entirely different state of things. As shown in the table, there was not a trace of precipitin reaction visible in the case of a 24 hour culture at a dilution of 1:4, nor was there in that of a 3 day culture. When undiluted, there was a good, but not a particularly heavy reaction, even with a precipitating serum of very high titer. Evidently in this case the precipitable substance does not dissolve out into the medium during the growth period. The amount of specific carbohydrate capable of reacting with homologous serum in a 1 to 3 day culture of either (a) or (b) is practically nil.

There remains the question of the relative amounts of precipitable material present in the two strains, the one capsulated and the other not, for this ratio is perhaps at this time the best chemical evidence obtainable of a relation between capsule and soluble specific substance. More crude material is obtainable from equal areas, for example, the surface of ten pie plates in the case of the capsulated strain. No count of the relative numbers of organisms involved has been attempted, but from the uncapsulated strain only about two-thirds as much crude material can be obtained per unit of culture surface. And this material is about 100 times *less active* with high titer precipitating serum (from either strain) than is similar material from the capsulated strain. No attempt has been made to prepare or purify a large amount of the "residue" from the mutant.

The simplest assumption is therefore that suggested by Mueller (8) and Heidelberger, Goebel, and Avery (9), that capsular material and soluble specific substance are identical. In the case of a morphological capsule, the specifically precipitable substance or "residue" probably is produced in much larger quantity and located peripherally Other substances, such as bacterial mucins, may also take part in the capsule formation. In the case of the capsulated *B. coli*, there was no indication of more than a trace of mucin. From the ease with which the material can be obtained in relatively pure condition, it is very unlikely that much is present. On the other hand, in the preparation of specific carbohydrates from a peculiarly viscid strain of Friedländer's bacillus (unpublished) there was obviously a *very*

large admixture of an impurity that was probably mucin, or something similar.

The substance obtained from the capsulated strain of the colon bacillus is not identical with any specific carbohydrate thus far already described. It is composed of 80 per cent of hexose, probably partly of dextro- and partly of levorotatory sugar, since the rotation of the hydrolysate is slight. It is precipitable by basic lead acetate and ferric chloride. It is rather lighter in weight and more readily soluble in distilled water than are some similar substances prepared by the writer from the Friedländer bacillus. The presence of glucuronic acid is indicated. Some evidence for a relation between capsule and specific substance was obtained. From equivalent areas of growth on agar in the case of the mutant about two-thirds as much material, as in the case of the viscid strain, precipitable with 1 volume of alcohol, can be obtained. This material, obviously very impure, is about 100 times *less* active with homologous serum than similar material from the capsulated strain. Although this cannot be interpreted as proving that the capsulated organism contains 100 times as much specific carbohydrate as the mutant, it is a very significant difference.

The specific substance of the capsulated colon strain does not diffuse out into the fluid culture media, as does that of the pneumococcus. There is so little present in the filtrate of a 24 hour culture, such as used in the original virulence tests, that it indicates that the substance functions distinctly as a capsule, a protecting layer, rather than in solution. Attempts to recombine the extract with living bacilli in testing virulence proved unsatisfactory, since non-specific additions in minute amounts also reduced the resistance of the peritoneal cavity of the guinea pigs.

SUMMARY AND CONCLUSIONS.

1. The soluble specific substance obtained from a capsulated strain of *B. coli* is not identical with any specific substance heretofore described. It is a carbohydrate, composed of 80 per cent of hexose, probably partly of dextro- and partly of levorotatory sugar, since the rotation of the hydrolysate is low. Glucuronic acid is probably present in the molecule.

2. Crude "residue" or specific substance obtained from the uncapsulated mutant was about 100 times *less* active with homologous serum than similar material from the capsulated strain. This supports the view that capsular substance and soluble specific substance are the same. In cases where there is a well marked capsule, the specific substance is probably produced in greater amount and located peripherally.

3. Capsular substance is probably significant for virulence when functioning as a morphological capsule. It is present in filtrates of young culture only in very small amounts.

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THE SURFACE COMPOSITION OF THE TUBERCLE BACILLUS AND OTHER ACID-FAST BACTERIA.

By STUART MUDD, M.D., and EMILY B. H. MUDD.

(*From the Henry Phipps Institute of the University of Pennsylvania, Philadelphia.*)

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The tubercle bacillus and other acid-fast bacteria are remarkable for their high lipin content, for their difficult but tenacious staining properties, and for their resistance to the ordinary destructive agencies of the body. It seemed natural in correlating these properties to postulate for the tubercle bacillus a difficultly permeable lipoid envelope[1] (Ehrlich) to which its tinctorial and biological peculiarities were ascribed. So simple a conception of the surface of the tubercle bacillus has become untenable in the light of recent investigations. Evidence will be presented in the present communications to indicate that the surfaces of the acid-fast bacteria are complex, containing at least lipin and protein, and quite likely also carbohydrate components; that these surfaces are profoundly modified as the result of interaction with serum; and that the acid-fast staining property is independent of the state of the surface.

Evidence for the Existence of Lipin in the Surface.

The most direct evidence for the presence of lipin in the surface of the acid-fast bacteria is the striking and characteristic ease with which they are wetted by oil or fatty acid. These microorganisms have been studied in oil-water interfaces in the manner already described for bacteria and blood cells,[2] and observations on more than 1000 such film preparations with modified and unmodified acid-fast bacteria have been recorded. Normal acid-fast bacteria grown on glycerol agar slants and suspended in 0.8 per cent sodium chloride

[1] Ehrlich, P., *Deutsch. med. Woch.*, 1882, viii, 269.

[2] Mudd, S., and Mudd, E. B. H., *J. Exp. Med.*, 1924, xl, 633, 647; 1926, xliii, 127. Mudd, S., *Proc. Soc. Exp. Biol. and Med.*, 1925–1926, xxiii, 569.

solution, certain mammalian tubercle bacilli excepted, pass spontaneously through the interface into the test oil, tricaprylin (Kahlbaum).

This typical spontaneous passage through the interface into oil has been observed repeatedly with the strains listed below.

M. tuberculosis: Bovine III, Bovine C, Bovine 816, and Bovine 960 (816 and 960 are isolations from tuberculous laboratory animals and probably the same strain as Bovine C); *M. avium:* Egret, Avian III, "lepra bacillus" of Kedrowsky,[3] homogeneous culture of Arloing;[3] "tubercle bacilli" of cold blooded animals: *M. chelonei, M. ranæ, M. marinum;*[4] saprophytes: *M. smegmatis,* "*pseudoperlsucht*"[5] bacillus, "milk" bacillus.

The behavior described for acid-fast bacteria is in contrast to that of other bacteria which are very stable in the interface,[2] and to blood cells[2] and spermatozoa,[6] all of which possess more or less stability in the oil-water interface and offer resistance to passage from the interface into the oil phase.

The bacteria were removed from the surface of glycerol agar slants with platinum wire and were suspended directly or with grinding in 0.8 per cent sodium chloride solution. They were then washed in 0.8 per cent sodium chloride and studied against an oil immediately, or after standing overnight or longer in sodium chloride solution. As the slowly advancing oil phase touched the margin of a clump of bacilli suspended or sedimented in the sodium chloride phase, the interface ran forward locally, the oil swiftly wetting the bacteria. If the bacilli were not too strongly coherent the clumps were dispersed explosively by the tension along the interface, and the bacteria flung violently into the oil phase. Such violent dispersion was characteristic of bacteria which can be suspended with comparative ease, *i.e.*, avian tubercle bacilli, the "tubercle bacilli" of cold blooded animals, "leprosy bacilli" of Kedrowsky, and an atypical bovine strain, Bovine III.[7,8] Loose clumps formed by sedimentation from an even bacterial suspension were similarly dispersed by the interfacial tension and flung into the oil. Suspensions of a virulent bovine tubercle bacillus, Bovine C,[8] which can only be made

[3] Furth, J., *J. Immunol.*, 1926, xii, 273.

[4] Aronson, J. D., *J. Infect. Dis.*, 1926, xxxix, 315.

[5] Incorrectly referred to as "*M. pseudotuberculosis*" in Reference 3 and in *J. Immunol.*, 1927, xiii, 115.

[6] Mudd, S., and Mudd, E. B. H., *Biochem. Z.*, in press.

[7] Smith, Theobald, *J. Med. Research*, 1913, xxviii, 91.

[8] Mudd, S., and Furth, J., *J. Immunol.*, 1927, xiii, 369.

with the aid of grinding, usually contain clumps so coherent as not to be dispersed by the interfacial stresses; the interface usually runs forward on such clumps and they pass entire into the oil.

As already indicated the preparations are ordinarily so made that the oil encroaches on the aqueous part of the film; the interface thus typically overtakes the bacteria suspended in the aqueous phase. Occasionally, however, the interface retraces its course for a time, thus overtaking bacteria which have already passed into the oil. Such bacteria do not pass back through the interface into the aqueous phase. On the contrary they are swept along before the retreating interface or may glide up or down in the interface. The oil-saline phase boundary is thus irreciprocally pervious to acid-fast bacteria; it permits spontaneous passage from saline to oil but not from oil to saline solution.

The four strains of human tubercle bacillus thus far studied, and one atypical bovine strain (B. C. G.), have differed from other acid-fast bacteria in not passing spontaneously into tricaprylin. These are the human strains H 37 (slightly virulent),[8] R 1 (avirulent),[8] Koch (avirulent),[8] P 15 B (highly virulent),[9] and the Pasteur Institute vaccine strain B. C. G.[10] All these strains have shown stability in the oil-saline interface; the degree of stability, however, is slight and not at all comparable to that of ordinary bacteria. Whether or not the slightly lesser oil miscibility[11] observed for these four human strains as compared to other acid-fast bacteria (B. C. G. excepted) is a characteristic property of the human type of tubercle bacillus we do not know. It is suggestive in this connection, however, that chemical evidence has seemed to indicate a higher wax but *lower fat* (*i.e.*, saponifiable lipin) content for human tubercle bacilli than for other acid-fast bacteria.[12]

[9] Perla, D., *J. Exp. Med.*, 1927, xlv, 215.

[10] Calmette, A., Guérin, C., Nègre, L., and Boquet, A., *Ann. Inst. Pasteur*, 1926, xl, 89.

[11] By "oil miscibility" in these communications we mean more precisely the property of being readily wet by oil.

[12] Frouin, A., *Compt. rend. Soc. biol.*, 1921, lxxxiv, 606. Long, E. R., and Campbell, L. K., *Am. Rev. Tuberc.*, 1922–23, vi, 636. Wells, H. G., De Witt, L. M., and Long, E. R., Chemistry of tuberculosis, Baltimore, 1923, Chapter i.

Against tributyrin or triolein and several other oils[2] the acid-fast bacteria have possessed slight stability in the interface. This was true also of a sample of tricaprylin from another source than Kahlbaum. This tricaprylin was not optically clean by dark-field illumination, however, as Kahlbaum's has been. With no oil was the degree of stability of the acid-fast bacteria comparable to that of ordinary bacteria.

The surfaces of acid-fast bacteria grown on other media than glycerol agar slants have not been studied with any thoroughness. A culture of *M. chelonei* and one of a "milk" bacillus on Long's[13] synthetic liquid medium, and cultures of *M. ranæ* and *M. chelonei* on glycerol bouillon, were found to pass spontaneously into tricaprylin in typical fashion. An occasional experiment has shown, however, that impurities in liquid menstrua may sometimes coat acid-fast bacteria, causing change of surface properties simulating those produced by sera (see paper following).

The Mechanism of Acid-Fast Staining.

Corroborative evidence of the existence of lipin in the surface of the acid-fast bacteria is furnished by the fact that the oil miscibility of the surface may be greatly decreased by extraction with alcohol. Tubercle bacilli (Strain Bovine III) were extracted with 95 per cent ethyl alcohol in a Soxhlet from 3 to 4 weeks by Dr. J. D. Aronson. Suspended in salt solution and studied with tricaprylin these extracted bacilli were highly stable in the interface, accumulated there in great numbers, and passed into the oil only with the expenditure of much mechanical work. Some "milk" bacilli were similarly extracted for 2 weeks and became stable in the interface. Both the extracted tubercle and "milk" bacilli retained their acid-fast staining properties. It is evident then that acid fastness does not depend upon the integrity of the surface membrane as originally postulated by Ehrlich.[1] A theory consonant with known facts has been suggested by Long.[14]

The surface lipin material can furthermore be coated over and the bacterial surface rendered oil-immiscible by sensitization with serum (see paper following). Such sensitized bacteria have shown normal acid-fast staining.

[13] Long, E. R., *Tubercle*, 1924, vi, 132.

[14] Wells, H. G., De Witt, L. M., and Long, E. R., Chemistry of tuberculosis, Baltimore, 1923, 101.

Evidence for the Existence of Protein in the Surface.

Evidence for the existence of protein in the surface has chiefly been derived by Freund[15] from study of the cataphoresis and agglutination of tubercle bacilli and their lipoid and protein components. Both cataphoresis and agglutination in any given circumstances are conditioned by the cell surface. Freund found that the behavior of the whole bacilli in several important respects resembled the behavior of extracted or "defatted" bacilli and that of protein extracts rather than that of the lipoid extracts. The isoelectric points found, for instance, were:

Whole tubercle bacilli, pH 3.0; defatted tubercle bacilli, pH 2.8; protein precipitated from watery extract, pH 2.8; lipoid prepared from alcoholic extract, pH 1.5; lipoid prepared from ether extract, pH 1.0.

The agglutination of the whole and defatted bacteria by electrolytes, moreover, resembled that of suspended protein and Gram-negative bacteria in that the higher concentrations of salts stabilized the suspension; the same salt concentrations did not prevent but indeed caused agglutination of the lipoid particles.[15]

The existence of the group of human tubercle bacilli above described as differing in their surface-wetting properties from other typical acid-fast bacteria favors, although it of course does not prove, the conception of the acid-fast bacterial surface as complex. The most probable explanation of this difference is a quantitative difference between the two groups in the amount of lipin and protein or other polar component of the bacterial surface.

Dr. Freund was originally inclined to regard the surface of tubercle bacilli as entirely protein,[15] and the writer as essentially lipoid. It is probably significant that Freund's cataphoresis experiments were performed with tubercle bacilli of human type. Human bacilli as already indicated have shown wetting properties differing less than those of other acid-fast bacteria from the properties of protein particles. Freund[16] and the writer are now agreed in regarding the surface of the acid-fast bacteria as complex.

A carbohydrate gum has been isolated from broth filtrates of

[15] Freund, J., *Am. Rev. Tuberc.*, 1925, xii, 124.

[16] Freund, J., personal communication.

human tubercle bacilli by Mueller[17] and by Laidlaw and Dudley from the bacilli themselves.[18] This carbohydrate in high dilution gives a specific precipitate with immune serum. It is known that the analogous carbohydrate haptenes in the pneumococci are components of the capsule.[19] It seems improbable that the carbohydrate haptene of the tubercle bacillus should be absent from the bacterial surface. Direct evidence bearing on this point is, so far as we know, however, entirely lacking.

SUMMARY.

Acid-fast bacteria in the boundary surface between salt solution and a test oil (tricaprylin) are spontaneously wet and enveloped by the oil. This behavior contrasts with that of all other cells studied by the interfacial tension method.

Four strains of human tubercle bacillus and an atypical bovine strain are an exception to the first statement above. These have possessed stability in the saline-oil interface; this stability is slight, however, and not comparable with that of non-acid-fast bacteria.

Acid-fast bacteria subjected to prolonged extraction with alcohol show resistance to wetting by oil comparable to that of non-acid-fast bacteria. These "defatted" bacteria nevertheless retain their acid-fast staining properties. Acid fastness cannot then depend on the integrity of a surface membrane.

Study of the cataphoresis of acid-fast bacteria by Freund has rendered the presence of protein in the surface highly probable. We are forced then to regard the surface of acid-fast bacteria as complex, containing at least lipoid and protein. Not improbably also carbohydrate is present.

[17] Mueller, J. H., *J. Exp. Med.*, 1926, xliii, 1, 9.
[18] Laidlaw, P. P., and Dudley, H. W., *Brit. J. Exp. Path.*, 1925, vi, 197.
[19] Heidelberger, M., *Physiol. Rev.*, 1927, vii, 123.

ON THE MECHANISM OF THE SERUM SENSITIZATION OF ACID-FAST BACTERIA.

By STUART MUDD, M.D., and EMILY B. H. MUDD.

(*From the Henry Phipps Institute of the University of Pennsylvania, Philadelphia.*)

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The peculiar surface properties of acid-fast microorganisms make them readily susceptible of direct study by the interfacial tension method, a procedure not heretofore applied to analysis of the interactions of bacteria and sera. The acid-fast bacteria are thus especially favorable material for study of the mechanism of serum sensitization.

I. Directly Observable Alterations in the Bacterial Surface Produced by Sensitization.

The surfaces of acid-fast bacteria grown on glycerol agar slants, and suspended in 0.8 per cent sodium chloride solution, as has already been pointed out, are usually vigorously wetted by a neutral oil, tricaprylin. When these bacteria are suspended in fresh sera and subsequently washed in salt solution and studied in a saline-tricaprylin interface, they are no longer easily wetted by the oil. They are on the contrary in equilibrium[1] in the oil-saline interface and pass into the oil only at the expense of mechanical work.

Strongly sensitized clumps of acid-fast bacteria may be carried ahead of the advancing oil phase, or, if the clump is adherent to the slide, it retards locally the advance of the oil and may even draw out the interface to form a "peninsula" or vacuole.[2]

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[1] Mudd, S., and Mudd, E. B. H., *J. Exp. Med.*, 1924, xl, 633, 647; 1926, xliii, 127.

[2] Similar phenomena have been described and figured for sensitized red blood cells (*J. Exp. Med.*, 1926, xliii, 127). However, the passage into the oil of clumps of sensitized acid-fast bacteria differs from that of sensitized erythrocytes in that no surface processes or "tails" are ever to be seen with the bacterial clumps.

Serum sensitization thus transforms the predominantly oil-miscible[3] surface of the acid-fast bacteria into a surface more readily wet by saline solution than by the oil. All stages of this transformation may be seen. A strong immune serum causes in low concentration just perceptibly decreased oil miscibility of its homologous microorganism. With increasing concentration of the sensitizing serum the resulting bacterial surface change usually increases to a maximum in the highest serum concentrations. Normal sera or immune sera with unrelated heterologous organisms cause detectable oil immiscibility in the higher concentrations only.

The *cohesion* of films of sensitized and unsensitized typhoid bacilli has been measured by Northrop and De Kruif; they found that, other factors being constant, the cohesion increased with the concentration of sensitizing immune serum, at least until maximum sensitization was reached.[4] This relation of sensitization to cohesion is very strikingly shown in the present experiments with acid-fast bacteria.

Suppose an even bacterial suspension virtually free of clumps to have been made, and equal portions mixed with serial dilutions of homologous immune serum. All tubes are centrifuged, the supernatant liquid decanted, and the sediment resuspended in salt solution. The sensitized bacteria resuspend in clumps whose size and coherence, as estimated from their resistance to dispersion by agitation, increase with the concentration of serum used for sensitization.[5,6] If now all tubes are centrifuged again and the sediment studied in the tricaprylin-saline interface the same relation of sensitization to cohesion is again brought out. The loose aggregates of unsensitized bacteria on contact with the advancing oil are explosively dispersed and flung into the oil. Weakly sensitized clumps are dispersed less completely, the dispersion occupies perceptible time, and the component bacteria are carried away from the clumps for greater distances along the the oil-saline interface before passing into the oil. Very strongly sensitized clumps may resist dispersion by the interfacial stresses altogether. In general, for any one bacterial suspension the degree of coherence of clumps produced by the sensitization and the degree of oil immiscibility produced by sensitization run roughly parallel.

The characteristic increase in resistance to wetting by oil produced by the serum sensitization of red blood cells and acid-fast bacteria has been called in an

[3] See Foot-note 11 in preceding paper.

[4] Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 664.

[5] Mudd, S., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiii, 569.

[6] Mudd, S., *J. Immunol.*, 1927, xiii, 113.

earlier paper[2] "a positive stabilization reaction." A more convenient term is "positive interface reaction."

To summarize, serum sensitization of the acid-fast bacteria causes two definite and directly observable changes in the bacterial surface:

1. A change from a surface readily wet by oil to a surface more readily wet by aqueous salt solution than by oil. This change is directly observed by microscopic examination of the bacteria in a saline-oil interface; thus detected, the surface alteration is said to constitute a "positive interface reaction."

2. An increased cohesiveness of the sensitized bacteria. This may be detected either by centrifuging the bacteria and then shaking up the sediment (resuspension reaction) or by microscopic observation of the clumps in the saline-oil interface.

II. Specificity of the Interface Reaction and Its Relation to Agglutinin Binding and Complement Fixation.

The procedure in obtaining the comparative titers given in this paper has been as follows:

The microorganisms were suspended in 0.8 per cent sodium chloride solution, with grinding when necessary, and the clumps thrown down by centrifugation. Portions of the relatively even supernatant suspension were mixed with serial dilutions of serum and put in the ice box overnight. The sera were unheated except where otherwise specified. The macroscopic *agglutination* readings were made in the morning. All tubes were centrifuged; the sediment was then either resuspended in the same serum dilutions, or else the serum was decanted and the sediment resuspended in a few drops of 0.8 per cent salt solution. The unsensitized bacteria resuspended evenly, the sensitized in clumps. This procedure has been termed the *resuspension* reaction. Its details and uses have been given in full elsewhere;[6] it is a simple and practical way of determining the binding of agglutinin by difficultly agglutinable strains. The bacteria were then washed once or more in salt solution and studied in the saline-tricaprylin *interface*. (Triolein was used as test oil in a few experiments.) Suspensions of the same strains and the same sera were used simultaneously by Dr. J. Furth in the *complement fixation* reaction.[7,8] The titer given is in each case unless otherwise indicated the highest dilution of serum which produced a perceptible effect.

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[7] Furth, J., *J. Immunol.*, 1926, xii, 273.

[8] Mudd, S., and Furth, J., *J. Immunol.*, 1927, xiii, 369.

The binding of antibody as detected by the interface reaction has been found to be serologically specific and to indicate antigenic relationships among acid-fast bacteria which correspond to those indicated by agglutinin binding, and, except for the anomaly mentioned in the next paragraph, to those indicated by complement

TABLE I.

Comparative Titers with a Saprophyte, an Avian and a Bovine Tubercle Bacillus, and Their Corresponding Antisera.

		Antiserum against "milk" bacillus	Antiserum against Arloing bacillus	Antiserum against Bovine III
Suspension of "milk" bacillus	Agglutination	1:640++*	1:40+*	1:40+*
	Resuspension	1:640 flocculi*	1:2.5	1:5+ (others?)
	Interface reaction	1:80	1:1.25+, 1:10−	1:2.5
	Complement fixation	1:1000		
Suspension of Arloing bacillus	Agglutination	1:80	1:640++*	1:80
	Resuspension	1:5	1:640 flocculi*	1:160
	Interface reaction	1:2.5−	1:640	1:80
	Complement fixation		1:500	1:100
Suspension of Bovine III	Agglutination	1:80+*	1:40	1:320
	Resuspension		1:20	1:160
	Interface reaction	1:5	1:5− or ∓	1:80
	Complement fixation	1:100	1:100	1:500

Moderate cross-reactions between *M. avium* (homogeneous culture of Arloing[7]) and *M. tuberculosis* (Bovine III strain). "Milk" bacillus practically unrelated serologically. All antisera from rabbits.

* Titer not reached.

fixation. The specificity of the interface reaction is illustrated by Tables I and II.

The anomaly just referred to is that certain sera homologous with such difficultly agglutinable strains as virulent mammalian tubercle bacilli may give higher interface and agglutination titers with heterol-

ogous than with homologous microorganisms. Examples of such anomalous titers are given in Tables III and IV.

This anomaly has occurred when the heterologous was closely related to and much more easily agglutinable than the homologous strain. The complement fixation titers in such instances have been normal, *i.e.* higher with the homologous than with the heterologous strain. The crossed combinations, *i.e.* antiserum for easily agglutinable strain with homologous easily agglutinable and heterologous difficultly agglutinable strain, have likewise been normal for interface and agglu-

TABLE II.

Comparative Titers with Three Species of "Tubercle" Bacilli of Cold Blooded Animals and Their Corresponding Antisera.

		M. marinum antiserum	*M. chelonei* antiserum	*M. ranæ* antiserum
M. marinum suspension	Agglutination	1:80	All negative	1:20
	Resuspension	1:320	All negative	1:2½
	Interface reaction	1:160	1:5	1:10
	Complement fixation	1:1000	1:20 negative	1:20 negative
M. chelonei suspension	Agglutination	1:40	1:1280*	1:80
	Resuspension	All negative	1:320	1:10
	Interface reaction	1:2½∓	1:80	1:5
	Complement fixation	1:50 negative	1:1000	1:100
M. ranæ suspension	Agglutination	1:5	1:10	1:2560*
	Resuspension	1:2½	1:10	1:2560*
	Interface reaction	1:2½	1:5	1:640
	Complement fixation	1:50 negative	1:50	1:2000*

Little or no serological relationship found between the three species. All antisera from rabbits.

* Titer not reached.

tination reaction as well as for complement fixation. Similar anomalies in agglutination experiments have been noted by Griffith[9] and by Wilson.[10] It may be necessary to use "absorption tests" in order to differentiate such strains with certainty.

The agglutination, resuspension, interface, and complement fixation titers have all been obtained for some forty-three combinations

[9] Griffith, A. S., *Tubercle*, 1924–25, vi, 417.

[10] Wilson, G. S., *J. Path. and Bact.*, 1925, xxviii, 1, 69.

TABLE III.

Comparative Titers with Virulent Bovine[8] (816) and Avirulent Bovine (III) Tubercle Bacilli, "Leprosy Bacillus" of Kedrowsky,[7] and Corresponding Antisera.

		Bovine 816 antiserum	Bovine III antiserum	"Leprosy bacillus" of Kedrowsky antiserum
Bovine 816 suspension	Agglutination	1:5		
	Resuspension	1:80		
	Interface reaction	1:10		
	Complement fixation	1:1000		
Bovine III suspension	Agglutination	1:400	1:400	1:800
	Resuspension	1:100	1:200	1:400
	Interface reaction	1:200	1:100	1:200
	Complement fixation	1:500	1:500	1:100±
"Leprosy bacillus" of Kedrowsky suspension	Agglutination	1:200+*	1:200	1:800+++*
	Resuspension	1:25	1:50	1:800+*
	Interface reaction	1:12½	1:12½ negative	1:800
	Complement fixation			1:1000*

Higher interface and agglutination titers with easily agglutinable strains Bovine III and "leprosy bacillus" in Bovine 816 antiserum than with difficultly agglutinable Bovine 816 in Bovine 816 antiserum. Cross-reactions between Bovine III and "leprosy bacillus" show ordinary specificity. All antisera from rabbits.

* Titer not reached.

TABLE IV.

Comparative Titers with Avian (Egret) and Human[8] (H 37) Tubercle Bacilli and Corresponding Antisera.

		Egret (avian) antiserum	H 37 antiserum
Egret (avian) suspension	Agglutination	1:400+++*	1:12.5+
	Resuspension	1:400 granular*	1:50
	Interface reaction	1:400+	1:12.5±
	Complement fixation	1:1000	1:20–50
H 37 human suspension	Agglutination	1:12.5±	1:12.5−
	Resuspension	Inconclusive	1:12.5
	Interface reaction	1:12.5−	1:12.5−
	Complement fixation	1:100	1:500

In H 37 antiserum agglutination and interface titers higher for egret strain than for H 37. Complement fixation shows ordinary specificity. Antisera from rabbits. Test oil triolein.

* Titer not reached.

of serum and bacterial suspension. The interface reaction proves to be the least sensitive, *i.e.* to have the lowest average titer. The ratios of the average titers of the several reactions to that of the interface reaction are:

Agglutination titer:interface titer::3.5:1
Resuspension titer:interface titer::2.4:1
Complement fixation titer:interface titer::13.6:1

The resuspension titer:interface titer ratio has been less inconstant than the others. Moreover in those cases already cited (for instance Tables III and IV) in which the agglutination and resuspension titers were anomalous and the complement fixation titers normal, the interface titers paralleled the agglutinin binding and not the complement fixation titers. Since, therefore, agglutination as estimated by the improved or resuspension method, cohesion as estimated by the resuspension and interface methods, and the bacterial surface change (decreased oil miscibility) detected by the interface reaction all show general parallelism, it seems safe to conclude that the bacterial surface change, like the other effects, is at least in considerable part due to the binding of agglutinins by the bacterial surface.

Similar decrease in surface oil-miscibility and increase in cohesion has been found as a result of serum sensitization of mammalian red blood cells, but in this case the surface change was correlated with the binding of hemolytic sensitizer and was relatively independent of agglutinin binding.[2]

The importance of the physicochemical nature of the bacterial surface itself in agglutination is particularly well shown with the acid-fast bacteria. It has already been pointed out that agglutination as ordinarily detected may be entirely absent under circumstances in which the resuspension and interface reaction show abundant agglutinin to have been bound by the bacterial surface.[6,11] Conversely, with readily agglutinable strains agglutination may be complete in tubes in which interfacial observation shows that only a part of the bacterial surface has been altered by deposit of antibody. The latter point is illustrated in Tables V and VIII.

[11] It has repeatedly been shown also that inagglutinable bacteria may adsorb agglutinins (Buchanan, R. E., *J. Bact.*, 1919, iv, 82).

TABLE V.

"Milk" Bacillus Suspension with Homologous Rabbit Antiserum.

	Final serum concentration						
	5 per cent	2.5 per cent	1.2 per cent	0.62 per cent	0.31 per cent	0.16 per cent	Control
Agglutination	Complete	Complete	Complete	Complete to +++	+++	++	Tr.
Resuspension	All bacteria are in one or more great masses				Even suspension with some gross flocculi	Even suspension with some gross flocculi	Even suspension
Interface reaction	++++	++	+	−	−		−

TABLE VI.

Resuspension and Interface Reactions with Sera of Normal Goats and Suspension of M. tuberculosis (Strain Bovine III).

Serum from	Final serum concentration															
	80 per cent		40 per cent		20 per cent		10 per cent		5 per cent		2.5 per cent		1.25 per cent		0.62 per cent	
	Resuspension*	Interface*	Resuspension	Interface	Resuspension	Interface	Resuspension	Interface	Resuspension	Interface	Resuspension	Interface	Resuspension	Interface	Resuspension	Interface
Goat 25, unheated	Coarse flocculi	++++	Coarse flocculi	+++	Fairly coarse flocculi	+++	Fine flocculi	++ or +		+ weak	Even suspension	+ weak	Even suspension	±	Even suspension	−
Goat 25, inactivated	Even suspension	+	Fine flocculi	±		−	Almost even suspension		Almost even suspension	−						
Angora goat unheated	Even suspension		Coarse flocculi	+++	Finer flocculi	++	Even suspension	+	Even suspension	−	Even suspension		Even suspension	−	Even suspension	
Angora goat inactivated	Even suspension	+ very weak	Almost even suspension	−			Even suspension									

* Bacteria washed after about 3 hours contact with serum; bacteria in other tubes in serum dilutions overnight.

TABLE VII.

Titrations of Human and Rabbit Sera against Human and Bovine Tubercle Bacilli, Virulent Strains P 15 B and Bovine C.

		Final serum concentration								
		80 per cent	40 per cent	20 per cent	10 per cent	5 per cent	2.5 per cent	1.25 per cent	0.62 per cent	Control
Human serum, Patient N. Br., with human bacteria	Agglutination	−	−	−	Slight tr.	Tr. to +	−	−	−	−
	Resuspension	Medium flocculi	Fine flocculi	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	
	Interface	+ weak	−					−	−	
Human serum, Patient N. Bl., with human bacteria	Agglutination	−	−	−	−	Tr. to +	−	−	−	−
	Resuspension	Fine flocculi	Fine flocculi	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	
	Interface	+	+ weak	+ weak	−					
Normal rabbit serum I with human bacteria	Agglutination	Slight tr.	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	Slight tr.	
	Resuspension	Medium flocculi	Medium to fine flocculi	Very fine flocculi to even suspension	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	
	Interface	++	++ to +	+	+	+ weak	−	−		
Normal rabbit serum II with human bacteria	Agglutination	−	Tr.	Tr. to +	+	+	− to tr.	− to tr.	−	
	Resuspension	Medium to fine flocculi	Fine flocculi	Very fine flocculi	Some fine flocculi	Some fine flocculi	Some fine flocculi	Even suspension	Even suspension	
	Interface	++	+	+	+ weak	+ weak	+	−	−	
Human serum, Patient N. Br., with Bovine C bacteria	Agglutination	Tr.	Tr.	Tr.	Tr. to +	+	− to tr.	− to tr.	−	
	Resuspension	Very fine flocculi	Fine flocculi	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension
	Interface	−	−				−		−	

Human serum, Patient N. Bl., with Bovine C bacteria	Agglutination	−	−	Tr.	−	Tr. to +	Tr.	Slight tr.	−
	Resuspension	Even suspension	Even suspension	Even suspension	Even suspension	Stringy bodies	Stringy bodies	Even suspension	Even suspension
	Interface	+	+	−					
Normal rabbit serum I with Bovine C bacteria	Agglutination	Tr.	+	+	Tr. to +	Tr. to +	−	Tr. to +	−
	Resuspension	Fine flocculi	Fine flocculi	Fine flocculi	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension
	Interface	++	++ to +	+	−				
Normal rabbit serum II with Bovine C bacteria	Agglutination	−	Tr. to +	Tr. to +	Tr.	+	+	Tr.	−
	Resuspension	Fine flocculi	Fine flocculi	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension
	Interface	+	+ weak	−					

Patient N. Br. pregnant and with four plus Wassermann at time blood was taken for tests tabulated. Chest negative.
Patient N. Bl. with negative Wassermann at time blood was taken and for at least a year previously. Chest negative.
Rabbits normal. Test organisms a virulent bovine tubercle bacillus, Bovine C, and a virulent human tubercle bacillus, P 15 B.

III. Effects of Sensitization with Normal Sera.

The bacterial surfaces are modified by treatment with fresh normal sera in a manner quantitatively less but qualitatively indistinguishable by the methods used, at least, from the effects of immune sera. Sensitization with normal sera decreases the oil-miscibility and increases the cohesion of the acid-fast bacteria, but to a less degree and with lower titer than homologous immune sera.

Heating sheep, goat, rabbit, and human sera for 30 minutes at 56°C. usually decreased but did not abolish their effect on the bacterial surface. Similar inactivation of guinea pig sera left them without detectable effect on the bacterial surface. The test organisms used with normal sera have been *M. tuberculosis:* Strains H 37 (human), P 15 B (human), Bovine III, and Bovine C; "butter" bacillus; and *M. chelonei* (turtle). A titration is given in Table VI.

The sera of nine patients from the syphilis clinic have been tested against Bovine III.

These patients with the exception of two cases of arrested pulmonary disease were without clinical tuberculosis. The Wassermann reaction on the same serum specimen was four plus in two cases, was and had been negative in one case, and was negative or weakly positive but had formerly been four plus in the other six cases. The final serum concentration used for sensitization was in each case 80 per cent, and the test oil in the interface reaction was triolein. The sensitized bacteria, as in all other cases, were washed before study in the interface.

All these sera caused the characteristic bacterial surface change before, and all with one exception after heating for 30 minutes to 56°C. Another human serum, that of a girl of 12, negative for tuberculosis and with negative Wassermann, gave a two plus interface reaction after heating.

Titrations of two human and two normal rabbit sera against a human and a bovine tubercle bacillus are given in Table VII. The test oil in this case was tricaprylin.

IV. The Agglutination Prezone.

Since the important work of Northrop and De Kruif,[12] it has been recognized that the main features of bacterial agglutination can be

[12] Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 639, 655.

formulated in terms of the repellent forces of the electrical charges on the bacteria, which tend to keep them apart, and the cohesive force which tends to hold the bacteria together once they have collided. Substances which reduce the charge below a certain critical level ordinarily cause agglutination unless the cohesive force is also reduced.

The prezone in specific bacterial agglutination has not fitted well into this formulation. Studies by cataphoresis have indicated that the negative bacterial charge is reduced to a minimum in concentrated agglutinating sera.[13] Fig. 2 of Shibley's paper of 1924[13] is particularly instructive in this connection. This shows the bacterial surface charge falling with increasing serum concentration until the critical level is reached and agglutination occurs; further concentration of

TABLE VIII.

Egret Avian Suspension with Antiegret Rabbit Serum.

	Final serum concentration						
	8 per cent	4 per cent	2 per cent	1 per cent	0.5 per cent	0.25 per cent	NaCl
Agglutination	+	+	++	+++	++++	+++	−
Interface reaction	++++	++++	+++ or ++	+	+	+	−

Agglutination shows prezone, interface reaction does not.

serum reduces the charge along the same curve but agglutination ceases.

That the prezone is not due to lack of cohesiveness of the bacterial surface in concentrated sera is shown by our own results. Tables VII and IX of this paper and Tables IV and VI of an earlier communication[6] show that spinning the sensitized cells in the centrifuge abolishes the agglutination prezone. Similarly interfacial observation of the sensitized washed bacteria shows that the characteristic change in surface properties increases to a maximum after treatment

[13] Wells, H. G., The chemical aspects of immunity, New York, 1925, 137; Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 655; Shibley, G. S., *J. Exp. Med.*, 1924, xl, 457; Freund, J., *Am. Rev. Tuberc.*, 1925, xii, 124; Falk, I. S., and Jacobson, M. A., *J. Infect. Dis.*, 1926, xxxviii, 182; Shibley, G. S., *J. Exp. Med.*, 1926, xliv, 674.

TABLE IX.

Bovine III Suspension with Homologous Goat Antiserum.

	Final serum concentration							
	8 per cent	4 per cent	2 per cent	1 per cent	0.5 per cent	0.25 per cent	0.12 per cent	0.6 per cent
Agglutination.....	+	+	++	+++	++++	+++	++	+
Resuspension.....	Very coarse flocculi	Very coarse flocculi	Flocculi	Flocculi	Flocculi	Even suspension	Even suspension	Even suspension
Interface reaction....	++++	++++	++++	++++	++ or +		+ weak	+ weak

Agglutination shows prezone; resuspension and interface reactions do not.

with concentrated sera and is without prezone (Tables VII to IX of this paper, and Table IV of a previous paper[6]).

Since the agglutination prezone then is not due to a failure of concentrated sera to reduce the bacterial surface charge or to increase the cohesiveness of the bacteria and may be abolished by centrifugation, we suggest that the prezone in such cases is due to interference with the collisions of the bacteria which are of course prerequisite to their clumping. Whether the interference with the colliding of the bacteria is chiefly due to increased viscosity of the menstruum, or to an accumulation of excess colloidal material in the bacteria-serum interfaces is uncertain; possibly both effects play a part.

It seems that the observations cited in the preceding paragraph are incompatible with the conception of these prezones as due to combination of the bacteria with Ehrlich's "agglutinoids."[14]

Krumwiede, Cooper, and Provost[15] have reported the production of prezones or the exaggeration of existing prezones by addition of material containing products of bacterial autolysis or media constituents. These workers also attribute their prezones to interference with agglutination by excess colloidal material.

V. Partial Reversibility of the Bacterial Surface Change.

Since the pioneer observations of Hahn and Trommsdorff[16] and Landsteiner[17] a number of investigators have demonstrated the possibility of recovering antibodies by partial dissociation of antigen-antibody combinations.[18] In the acid-fast group Aronson[19] has reported partial recovery of complement-fixing antibodies from combination with *M. tuberculosis*, or with alcoholic or saline extracts of tubercle bacilli. It seemed probable therefore that subjecting the sensitized bacteria to conditions that have been described as favoring dissociation might cause alteration of the bacterial surface in the

[14] Park, W. H., and Williams, A. W., Pathogenic microörganisms, Philadelphia and New York, 8th edition, 1924, 192.

[15] Krumwiede, C., Cooper, G., and Provost, D. J., *J. Immunol.*, 1925, x, 93.

[16] Hahn, M., and Trommsdorff, R., *Münch. med. Woch.*, 1900, xlvii, 413.

[17] Landsteiner, K., *Münch. med. Woch.*, 1902, xlix, 1905; Landsteiner, K., and Jagić, N., *Münch. med. Woch.*, 1903, l, 764.

[18] Huntoon, F. M., *J. Immunol.*, 1921, vi, 117.

[19] Aronson, J. D., *Nat. Tuberc. Assn. Tr.*, 1922, xviii, 517.

direction of the normal. This expectation has been verified by experiment, and further evidence has thus been furnished that the changes observed to follow sensitization are consequent on combination of antibodies with materials in the bacterial surfaces.

Serial dilutions of serum were mixed with bacterial suspension and allowed to stand in the ice box overnight. The sensitized bacteria were then washed, resuspended in saline, and studied in the interface. Into certain tubes of the series 0.1 per cent Na_2CO_3[19] was distributed and into the other, usually alternate, tubes 0.8 per cent NaCl. Since we have not found evidence of appreciable dissociation by washing in NaCl, these saline tubes served as controls for the alkali-treated bacilli.[20] All tubes were returned to the ice box for five days or more. The tubes were then centrifuged, decanted, the sediment washed in NaCl, and studied in the interface.

Of ten series of sensitized bacteria prepared as described, six showed alteration of surface properties toward the normal as a result of treatment with Na_2CO_3. Results in the other four series were doubtful. Five of the positive instances are given in Table X.

Results of tests by resuspension were similar to those with the interface reaction. The sensitized bacteria digested with sodium carbonate and subsequently washed and resuspended in sodium chloride, were regularly in finer clumps than those in the alternate tubes digested with sodium chloride, as illustrated in Table XI. This result it is true is of less significance than the very convincing dissociation of antibody detected by the interface reaction, because the alkali might conceivably have dispersed the bacterial clumps simply through increasing the negative electric charge of the material on the bacterial surface. However, the only mechanism that, to our mind at least, satisfactorily explains both the observed interface and resuspension results after sodium carbonate treatment is dissociation of antibody from the bacterial surface.

VI. What Chemical Groups Are Responsible for the Bacterial Surface Change?

The evidence already cited indicates clearly, we believe, that fat-soluble material in the bacterial surface is coated over by the sensitiz-

[20] Indeed the impression has been gained that bacteria either normal or sensitized kept 24 hours or longer in NaCl tend to become slightly less oil-miscible.

ing serum antibodies or possibly by serum substances secondarily bound by these antibodies.[21] This shows that the actual chemical

TABLE X.

Dissociation of Antibodies by Sodium Carbonate with Return of Bacterial Surface toward Normal.

Tubes	Sensitized bacteria before treatment	Sensitized bacteria after treating with Na_2CO_3	Sensitized bacteria after treating with NaCl
A 1	++++	±	
A 2			+++
A 3	++	−	
A 4			+
B 1	++	+	
B 2	++ to +		++
C 1	++	+	
C 2	+	−	
D 1	++++	++	
D 2			+++
E 1	++	+	
E 2			++
E 3	++ to +	∓	
E 4			+

+ to ++++ indicate degrees of positive interface reaction. − indicates a normal (unsensitized) bacterial surface.

Series A (Bovine C), *M. tuberculosis* sensitized with homologous rabbit antiserum; serum concentration in Tube A 1, 20 per cent, in A 2, 10 per cent, etc.

Series B (Bovine III), *M. tuberculosis* sensitized with homologous goat antiserum; serum concentration in Tube B 1, 10 per cent, in B 2, 5 per cent.

Series C, *M. ranæ* sensitized with normal horse serum; serum concentration in Tube C 1, 80 per cent, in Tube C 2, 40 per cent.

Series D, *M. tuberculosis* (Bovine C) sensitized with homologous rabbit antiserum; serum concentration in Tube D 1, 20 per cent, in D 2, 10 per cent.

Series E, *M. tuberculosis* (Bovine C) sensitized with homologous rabbit antiserum; serum concentration in Tube E 1, 5 per cent, in E 2, 2.5 per cent, etc.

[21] It is evident from the parallel complement fixation studies described in Section II above that complement is bound by the sensitized bacteria. To what extent this complement material contributes to the observed bacterial surface change is uncertain. Even from inactivated sera there is evidence to indicate

group[22] which combines with the antibody responsible for the interface reaction is at least very closely associated with fat-soluble material in the bacterial surface. It is not clear from these data, however, whether the binding group is a part of the lipin molecule, is conjugated with it, or is merely in very close physical association with the lipin.

Kurt Meyer[23] showed that alcohol extracts of the tubercle bacillus make active "antigens" for the complement fixation reaction. Wadsworth, Maltaner, and Maltaner[24] prepared extracts of tubercle bacilli with various fat solvents

TABLE XI.

Dissociation of Antibody by Treatment with Sodium Carbonate. Resuspension Reactions.

	Final serum concentration			
	80 per cent	40 per cent	20 per cent	10 per cent
Sensitized bacteria before treatment	Medium flocculi	Medium flocculi	Medium flocculi	Medium flocculi
After treatment with Na_2CO_3	Almost even suspension		Even suspension with some medium flocculi	
After treatment with NaCl		Medium flocculi		Medium flocculi

M. tuberculosis (Bovine III) sensitized with serum of goat infected with Bovine C.

and found the acetone-insoluble lipoids to be potent complement fixation "antigens." Pinner has lately reported alcohol-soluble, acetone-insoluble sub-

that mid-piece (globulin) may be bound (Dean, H. R., *Proc. Roy. Soc. London, Series B*, 1911–12, lxxxiv, 416; Leschly, W., *Z. Immunitätsforsch., Orig.*, 1916, xxv, 219).

[22] We do not know how many antigens or antibodies are concerned in the sensitization process. The singular or plural nouns are used in this connection without implication.

[23] Meyer, K., *Z. Immunitätsforsch., Orig.*, 1912, xiv, 359; xv, 245.

[24] Wadsworth, A., Maltaner, F., and Maltaner, E., *J. Immunol.*, 1925, x, 241.

stances from tubercle bacilli to be active "antigens" in complement fixation and biologically active antigens when injected into rabbits.[25]

Freund, by agglutination and cataphoresis experiments, has demonstrated the possibility of sensitizing by serum the particles obtained by alcohol extraction of the tubercle bacillus.[26] This strongly suggests that a binding group is at least in some sort of chemical combination with the lipin.

Furth and Aronson[27] have shown 95 per cent ethyl alcohol extracts of a considerable number of acid-fast bacteria to contain serologically specific "antigens" for complement fixation. The writer has made some simple chemical tests with the extracts used by Furth and Aronson with the hope of gaining further light on the relation of the binding groups to the lipin.[28]

Amino acid tests with the material in these alcohol extracts were unsatisfactory. Millon's reaction was negative with suspensions of the residues from evaporation of the extracts. The possibility of traces of protein being present was not, however, satisfactorily excluded.

Molisch reactions with watery suspensions of the residues of these crude alcohol extracts were in all cases strongly positive. On hydrolysis positive orcinol-HCl tests for pentose were obtained.

The specific carbohydrate haptene of Mueller[29] and of Laidlaw and Dudley[30] likewise yields a pentose. The pentose in the alcohol extracts might conceivably be of nucleic acid origin. Nevertheless, finding such a carbohydrate and lipin in association in these serologically active and specific extracts suggests the possibility of harmonizing conflicting views in the literature on "lipoidal antigens." Possibly the "*lipoidal antigens*" of Meyer, Wadsworth, Maltaner, and Maltaner, Pinner, Freund, Dienes, and others and the surface lipoid material involved in the interface reaction *may be conjugated lipins owing their serological specificity to a carbohydrate haptene.* Such an

[25] Pinner, M., *Am. Rev. Tuberc.*, 1925, xii, 142.

[26] Freund, J., *Am. Rev. Tuberc.*, 1925, xii, 124 (Tables 8 to 12).

[27] Furth, J., and Aronson, J. D., *J. Immunol.*, 1927, xiii, 265.

[28] Thanks are due these workers for the material and to Professor D. W. Wilson for aid in studying it.

[29] Mueller, J. H., *Proc. Soc. Exp. Biol and Med.*, 1924–25, xxii, 209; *J. Exp. Med.*, 1926, xliii, 9.

[30] Laidlaw, P. P., and Dudley, H. W., *Brit. J. Exp. Path.*, 1925, vi, 197.

hypothesis at least is free from the *a priori* objections to lipoid antigens on the basis of their chemical simplicity.[31]

Dienes and his coworkers prepared an active alcohol extract of the tubercle bacillus. Partial separation from the lipoids increased the potency of the active part which they regarded as possibly identical with the carbohydrate haptene of Mueller and Laidlaw and Dudley.[32] Later this active non-lipoid group was interpreted as a haptene distinct from that of Mueller and Laidlaw and Dudley.[33]

It is of course well established that the tubercle bacillus contains protein antigenic material,[34] but with this we are not concerned in this study.

The Sensitizing Material.—It is common knowledge that the antibodies are intimately associated with the serum globulins and accompany the globulins through various chemical manipulations. Although considerable progress has recently been made by Felton[35] in defining the relations of antibodies to the globulin fractions, it remains uncertain still whether the antibodies are special sorts of globulins or are separate chemical substances attached to the globulins. However this may be, evidence has most recently been advanced by Shibley[36] to indicate that the binding of agglutinin by various bacteria gives them a surface coating of globulin. Shibley's figures indicate that globulin particles and bacteria which had fixed agglutinin from concentrated homologous antisera behaved identically in an electric field. That the globulin condensed on the bacterial surface is denatured is indicated both by analogy with the properties of other proteins condensed on surfaces and by the precipitation reactions of the sensitized bacteria themselves.

Through the kindness of Dr. L. D. Felton we have been able to compare the interfacial behavior of antipneumococcus globulins with the interfacial behavior of sensitized bacteria.

[31] Heidelberger, M., *Physiol. Rev.*, 1927, vii, 107.

[32] Dienes, L., and Schoenheit, E. W., *Proc. Soc. Exp. Biol. and Med.*, 1925–26, xxiii, 106.

[33] Dienes, L., and Freund, J., *J. Immunol.*, 1926, xii, 137.

[34] Wells, H. G., De Witt, L. M., and Long, E. R., Chemistry of tuberculosis, Baltimore, 1923, 73.

[35] Felton, L. D., *J. Infect. Dis.*, 1925, xxxvii, 199, 309.

[36] Shibley, G. S., *J. Exp. Med.*, 1926, xliv, 667.

A sample each of Type I, II, and III water-insoluble antipneumococcus horse serum protein which protects, agglutinates, and gives the rest of the immunological reactions,[37] was sent us by Dr. Felton. This material had been precipitated four times at the isoelectric point of the protein containing the protective substance and repeatedly washed with water.[37] As received the protein was suspended in distilled water in small aggregates of particles. The particles were about the size of bacteria and the aggregates in the dark field closely resembled small clumps of agglutinated microorganisms.

These globulins with immune properties were studied in the interface between distilled water and tricaprylin and between salt solution and tricaprylin. The saline suspension was prepared by cautiously adding 0.8 per cent sodium chloride solution to the protein in distilled water until the resulting mixture was only slightly cloudy. Much of the globulin was of course dissolved in so doing. The remaining aggregates did not scatter light so well as did the particles in distilled water; on contact with the interface they were quickly dispersed in the boundary plane; the resulting particles were visible for a moment spreading along the interface but were soon themselves dispersed until no longer visible. When a large aggregate was overtaken by a swiftly moving interface some of the protein dragged through into the oil with slight local retardation of the advancing interface.

In distilled water-tricaprylin interfaces, the globulin aggregates were in equilibrium. Large free floating aggregates were swept before the advancing oil with only that part of their surface which was contiguous to the boundary plane in the interface. Very small aggregates entered the interface and slid along it, but even with these the greater part of their mass was in the water rather than the oil. Small clumps adherent to the glass passed through the advancing interface, the latter being retarded locally; the aggregates were somewhat spread in passing through the boundary plane. Small clumps did not form peninsulas or vacuoles.

Globulin samples were denatured by bringing to a boil. The resulting soft coagula were broken up with glass rods and the flocculi studied in the distilled water-tricaprylin and saline-tricaprylin interfaces. The cohesion of the aggregates, their adhesion to the glass, and their resistance to passage into the oil were all plainly increased by denaturation. Even small aggregates were tightly stuck to the glass and passed through the interface without spreading but with formation of peninsulas and vacuoles.

Results with the globulins are summarized in Table XII.

It is to be noted that the changes in these globulins produced by heat denaturation were similar to those produced by heat injury of polymorphonuclear leucocytes and blood platelets.[2] This result is contrary to the expectation expressed in the earlier paper.

[37] Felton, L. D., personal communication.

The behavior of denatured globulin particles in the saline-tricaprylin interface and that of maximally sensitized acid-fast bacteria in such interfaces have been closely similar. Indeed we could not detect differences. This evidence is in harmony with the conclusion proposed by Shibley on the basis of cataphoretic and precipitation data that bacteria maximally sensitized with agglutinating serum are coated with denatured globulin. Much more extensive test of this important generalization is desirable.

TABLE XII.

Interfacial Behavior of Globulins with Immune Properties.

	Cohesion of globulin	Adhesion to glass	Resistance to passage from aqueous to oil phase	Behavior of aggregates in interface
Native globulin in NaCl solution	Slight	Slight	Slight	Aggregates are dispersed along interface
Native globulin in distilled water	Moderate	Moderate	Moderate	Aggregates at oil-water boundary largely in water; are somewhat spread in passing through interface
Denatured globulin in NaCl or distilled water	Great	Great	Great	Aggregates at oil-water boundary almost entirely in aqueous phase. No spreading of aggregates by interface

The interfacial behavior of the denatured globulins in saline solution was indistinguishable from that of maximally sensitized acid-fast bacteria in saline solution.

SUMMARY.

Serum sensitization of the acid-fast bacteria causes two definite and directly observable changes in the bacterial surface:

1. A change from a surface readily wet by oil to a surface more readily wet by aqueous salt solution than by oil. This change is observed by microscopic examination of the bacteria in a saline-oil interface; thus detected, the surface alteration is said to constitute a "positive interface reaction."

2. An increased cohesiveness of the sensitized bacteria. This may be detected either by centrifuging the bacteria and then shaking up

the sediment (resuspension reaction), or by observation of the clumps in the saline-oil interface.

The interface reaction is serologically specific and confirms the existence of qualitative differences among acid-fast bacteria.

The interface reaction parallels the binding of agglutinins as detected by the resuspension reaction, but not agglutination as ordinarily tested for. The interface reaction is less sensitive,—*i.e.*, gives lower titers—than the resuspension reaction in about the average ratio of 1:3. The interface reaction in most instances runs approximately parallel to the complement fixation reaction; under at least one set of conditions, however, the interface reaction is correlated with the binding of agglutinin but not with the complement fixation reaction. How much of the bacterial surface must be covered with agglutinin in order to produce agglutination varies greatly with the bacterial strain used.

The bacterial surfaces are modified by treatment with fresh normal sera in a manner quantitatively less but qualitatively not observably different from the effects of immune sera.

Heating normal human, sheep, goat, or rabbit sera for 30 minutes at 56°C. has usually diminished but not abolished their effect on the bacterial surface. Similar inactivation of guinea pig sera left them without detectable effect on the bacterial surface.

The agglutination prezone is shown to be due to interference by excess colloidal material with the collisions of the bacteria prerequisite to clumping. The prezone may be abolished by centrifugation and resuspension of the sediment.

Antibodies may be partially dissociated from the sensitized bacteria by alkali, with return of the bacterial surface toward its normal, unsensitized condition.

A carbohydrate yielding on hydrolysis a positive pentose test has been detected in the specific alcohol extracts of acid-fast bacteria studied by Furth and Aronson.[27] The tentative suggestion is made that the alcohol-soluble antigens of acid-fast microorganisms may be conjugated lipins owing their specificity to carbohydrate haptenes.

Protective antipneumococcus globulins after heat denaturation have shown behavior in the saline-tricaprylin interface indistinguishable from that of maximally sensitized acid-fast bacteria. This strengthens the evidence suggesting that sensitized bacteria are coated with denatured globulin.

IMMUNIZATION EXPERIMENTS WITH LECITHIN.

By P. A. LEVENE, M.D., K. LANDSTEINER, M.D., AND J. VAN DER SCHEER.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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In the case of Forssman's heterogenetic antigen it was found possible to incite the production of antibodies by the injection of its specific component along with proteins. In a similar manner immune bodies were obtained with alcoholic extracts of blood corpuscles (1) and of organs (2). Since it is assumed generally though not proven that the specific part of the heterogenetic antigen is of lipoid nature it was natural to test the immunizing properties of lipoids of known chemical constitution. This was undertaken by Sachs and Klopstock (3) with lecithin and cholesterol. Indeed these authors state that they obtained antibodies by injecting into rabbits emulsions of lecithin, or cholesterol, containing pig serum. The tests were carried out mainly by means of complement fixation. A confirmatory paper was published by Ornstein (4) who also reported on successful immunizations with cephalin and with cerebrosides.

There are some points in the observations of Sachs and Klopstock not easily understood on the basis of their assumptions. Their antilecithin serum reacted on lecithin Merck but it also reacted on cholesterol and even more intensely on the latter than on a certain lecithin preparation of higher purity than lecithin Merck. This phenomenon is ascribed by Sachs and Klopstock to the presence of some cholesterol in the injected antigen but against such a view may be pointed out that in the experiments of these authors it was rather difficult to produce sera which react upon cholesterol, by injections of this substance. Another difficulty arises from the fact that the purer of the two lecithin preparations employed was the less active. The explanations for these peculiarities offered by Sachs and Klopstock do not settle conclusively the questions at issue. Since the production of antibodies for well known lipoids would be of great

significance, it seemed desirable to us to repeat the experiments with various lecithins prepared by ourselves.

EXPERIMENTAL.

We injected three lots of five rabbits each, with ox brain lecithin, egg lecithin and hydro egg lecithin, respectively. In addition sera were prepared with a commercial egg lecithin preparation (Merck) as used by Sachs and Klopstock.

Lecithin Preparations.—Lecithin No. 1. Alcoholic extract of egg yolk was treated with a 25 per cent solution of cadmium chloride in methyl alcohol, the precipitate extracted twice with ether and decomposed with methyl alcohol containing 25 per cent of ammonia. The solution was concentrated and the residue was extracted with cold alcohol. The lecithin was again precipitated as a cadmium salt and the latter was repeatedly washed with ether. The cadmium salt was then treated with methyl alcohol containing ammonia gas; the solution was concentrated, taken up in a minimum quantity of ether and precipitated with acetone.

Analysis: C 64.6; H 10.49; N 2.17; P 3.96; amino N 0.

Lecithin No. 2. 15.0 gm. of lecithin No. 1 were dissolved in methyl alcohol, acetone was added until a sample on cooling to −5°C. showed the formation of a precipitate. The entire solution was then brought to a temperature of −5°C. and the precipitate formed was removed by centrifugalization. The mother liquor was concentrated nearly to dryness, the residue was taken up in ether and the solution was poured into an excess of acetone. The yield was 11.0 gm. This material was dissolved in 20.0 cc. of methyl alcohol, 20.0 cc. of water were added and the solution was adjusted to pH 4. Acetone was added so long as a precipitate formed. The yield was 9.0 gm.

Analysis: C 65.3; H 10.61; N 2.00; P 4.12.

Egg Lecithin No. 3. The cadmium salt, prepared as No. 1, was extracted with ether 8 times. The lecithin obtained in this manner was further purified as follows: 26.0 gm. of lecithin were dissolved in 40.0 cc. of ether, 40.0 cc. of 10 per cent acetic acid were added, the mixture was shaken for 1 hour and the lecithin precipitated with 500 cc. of acetone.

Analysis: C 66.00; H 10.59; N 2.03; P 3.90; amino N 0.

Brain Lecithin. The cadmium salt was decomposed as usual, the filtrate was concentrated, and taken up in ether; acetone was added until a small precipitate formed. This was removed by filtration. The filtrate was concentrated nearly to dryness, the residue was taken up in ether, acetone was added to the solution to incipient opalescence. The solution was chilled to approximately −8°C. A precipitate formed which was removed by centrifugalization. The mother liquor was concentrated almost to dryness and taken up in a little ether. Acetone was added to opalescence and the solution brought to about −20°C. A precipitate formed, which was separated by centrifugalization. It was then extracted with

acetone and dried under diminished pressure. It was preserved in an atmosphere of nitrogen gas.

Hydrolecithin. This was prepared from egg lecithin by reduction with hydrogen and colloidal palladium as a catalyst.

Analysis of Merck lecithin: C 65.41; H 10.55; N 1.81; P 3.41; NH_2-N 0.

Tests were made also with a number of other samples of lecithin prepared in the laboratory with various modifications of the above described methods.

Immunization.—Rabbits were selected the sera of which gave no reactions in flocculation and complement fixation tests with emulsions of cholesterolized alcoholic beef heart extract[1] and of Merck's egg lecithin. 240 mg. of lecithin were emulsified with 15 cc. of saline and 3 cc. of pig serum diluted to 15 cc. with saline were added. This emulsion was kept at room temperature for 1 hour before injection.

The rabbits received intravenous injections of 5 cc. of the lecithin emulsion generally at intervals of 3 to 5, sometimes 7 days. The sera were tested several times during the course of the experiments, with both the lecithin used for injection and Merck's egg lecithin.

Tests. The complement fixation tests with the egg, brain and hydrolecithins were carried out as follows: To 0.25 cc. of progressively doubled dilutions of the inactivated serum starting with a dilution 1:5 were added 0.25 cc. of an emulsion of lecithin (prepared by fairly rapid addition of 24 cc. of saline to 1 cc. of a ½ per cent solution of lecithin in alcohol) and 0.25 cc. of guinea pig serum diluted 1:10. After incubation at 37°C. for 1 hour 0.25 cc. of sheep blood immune serum (2½–3 units) and 1 drop of a 50 per cent sheep blood suspension were added.

For the flocculation tests 1 part of a ½ per cent alcoholic solution of the egg lecithin was emulsified by fairly rapid addition of 5 parts of saline. 0.2 cc. of the emulsion was added to 0.2 cc. of the inactivated serum diluted 1:2 and the readings were taken after 20 hours standing at room temperature. For the flocculation tests with the brain lecithin and hydrolecithin a different procedure was adopted since emulsions prepared by the method described for egg lecithin were very unstable and were flocculated by most normal rabbit sera. 1 part of a ½ per cent alcoholic solution of brain lecithin was added rapidly to 5 parts of distilled water and 0.2 cc. of this liquid was mixed with 0.2 cc. of the inactivated serum diluted twice with a 2.7 per cent salt solution. In the case of the hydrolecithin 1 part of a ½ per cent solution of the substance was added by drops to 5 parts of boiling distilled water. The emulsion was filtered hot. For the test the serum was diluted with distilled water instead of saline.

The strength of the reactions in the tests is indicated as follows: Complement fixation tests—0 = no hemolysis, tr = trace, w = weak, d = distinct, str = strong, vstr = very strong, ac = almost complete, c = complete hemolysis. Flocculation tests—0 = no flocculation, ftr = faint trace, tr = trace; ±, +, ++, +++, etc.

[1] For the technique see: *J. Exp. Med.*, 1927, xlv, 467.

By immunizing with egg lecithin Merck we obtained four strongly and two weakly active sera among six rabbits after six injections.

The sera obtained with brain lecithin and hydrolecithin gave no distinct flocculation or complement fixation with these preparations

TABLE I.

Number of sera	Injections made with:	Flocculation with emulsions of:			
		Merck egg lecithin	Merck egg lecithin freed from cholesterol	Lecithin No. 1	Lecithin No. 1 with addition of 12 per cent cholestorol
966	Egg lecithin No. 1	0	0	0	tr
967	" " " 1	+±	0	0	+
968	" " " 1	0	0	0	tr
969	" " " 1	0	0	0	0
970	" " " 1	0	0	0	0
810	Merck's egg lecithin	+++	++±	0	±
Normal rabbit No. 1		0	0	0	tr
Normal rabbit No. 2		0	0	0	±

TABLE II.

Numbers of sera	Injections made with:	Complement fixation with an emulsion of egg lecithin No. 1	Complement fixation with an emulsion of Merck egg lecithin
966	Egg lecithin No. 1	c,c,c,c,c	c,c,c,c,c
967	" " " 1	c,c,c,c,c	0,tr,c,c,c
968	" " " 1	c,c,c,c,c	c,c,c,c,c
969	" " " 1	c,c,c,c,c	c,c,c,c,c
970	" " " 1	c,c,c,c,c	c,c,c,c,c
809	Merck's egg lecithin	c,c,c,c,c	0,0,0,0,ac,c
810	" " "	vstr,ac,c,c,c	0,0,0,0,0,ac,c
Normal rabbit		c,c,c,c,c	c,c,c,c,c

or with Merck's egg lecithin after twelve injections. Occasionally weak flocculations were noticed but after further injections the reactions disappeared.

The tests with the sera taken after twelve injections of egg lecithin No. 1 are presented in Tables I and II. Complement fixation tests

TABLE III.

Emulsions used for the tests:

A. 24 cc. of saline were added fairly rapidly to 1 cc. of a ½ per cent alcoholic solution of egg lecithin No. 1. This preparation gave no Liebermann reaction for cholesterol.

B. This emulsion was made as A, with 1 cc. of a ½ per cent alcoholic solution of egg lecithin No. 1 to which had been added 0.06 cc. of a 0.25 per cent alcoholic solution of cholesterol, corresponding to 3 per cent of the weight of lecithin.

C. Emulsion made as in A, with 1 cc. of egg lecithin No. 1 to which had been added 0.06 cc. of a 1 per cent alcoholic solution of cholesterol, corresponding to 12 per cent of the weight of lecithin.

D. As A, with Merck's egg lecithin. This preparation was found by the Liebermann test to contain 1.5 per cent of cholesterol or less.

E. As A, with Merck's egg lecithin from which the cholesterol was removed by dissolving 2 gm. in 15 cc. of ether and reprecipitation with 30 cc. of acetone. This purification was repeated twice. The product gave no Liebermann reaction.

F. 1 cc. of a ½ per cent alcoholic solution of cholesterol was slowly added to 24 cc. of boiling distilled water and the emulsion was filtered hot (method of Keeser (5)). The dilution of the sera was made with 1.8 per cent salt solution.

Number of sera	Injections made with:	Complement fixation with emulsions of:					
		Egg lecithin No. 1 A	Egg lecithin No. 1 + cholesterol B	Egg lecithin No. 1 + cholesterol C	Merck's egg lecithin D	Merck's egg lecithin after removal of cholesterol E	Cholesterol F
967	Egg lecithin No. 1	c,c,c,c,c	c,c,c,c,c	ac,c,c,c,c	0,0,ac,c,c	0,0,ac,c,c	0,0,d,ac,c
809	Merck's egg lecithin	c,c,c,c,c	c,c,c,c,c	str,c,c,c,c,c	0,0,0,0,str,c	0,0,0,w,c,c	0,0,0,0,d,ac,c
810	" "	vstr,ac,c,c,c	ac,c,c,c,c	d,vstr,c,c,c,c	0,0,0,0,0,str,ac	0,0,0,0,w,c,c	0,0,0,0,0,str,ac
Normal rabbit No. 1		c,c,c,c,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c
Normal rabbit No. 2		c,c,c,c,c	ac,c,c,c,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c

performed after five injections were negative as well as flocculation tests at various other times.

The serum No. 967 which gave moderate reactions, as can be seen from Tables I and II, was tested against emulsions differing in their cholesterol content in comparison with sera prepared with Merck's egg lecithin (Table III).

The highly active sera resulting from the injection with lecithin Merck gave uniformly negative tests by the method of complement

TABLE IV.

Immune sera prepared by injections with Merck's egg lecithin	Complement fixations with emulsions prepared by addition of 24 parts of saline to a 1/2 per cent alcoholic solution of:			
	Merck's egg lecithin	Egg lecithin No. 1	Egg lecithin No. 2	Egg lecithin No. 3
No. 809	0,0,0,0,d,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c
" 810	0,0,0,0,0,tr,c	vstr,c,c,c,c	ac,ac,c,c,c	ac,c,c,c,c
" 811	0,0,0,0,ac,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c
" 812	0,0,ac,c,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c

Immune sera prepared by injections with Merck's egg lecithin	Flocculations of emulsions made by addition of 5 parts of saline to 1 part of a 1/2 per cent alcoholic solution of:			
	Merck's egg lecithin	Egg lecithin No. 1	Egg lecithin No. 2	Egg lecithin No. 3
No. 809	+++	±	0	0
" 810	+++	tr	ftr	ftr
" 811	++±	±	tr	0
" 812	+±	+	0	tr
Saline control	0	0	0	0

fixation with our egg lecithin preparations 1, 2 and 3. In the flocculation tests preparations 2 and 3 reacted faintly, No. 1 somewhat better, but considerably weaker than lecithin Merck (Table IV). The results with brain lecithin and hydrolecithin were similar. Some other of our preparations gave more distinct flocculation with the Merck lecithin immune sera but practically negative complement fixation as far as they were examined.

SUMMARY.

In testing several egg lecithin preparations prepared by ourselves it was found that they did not react in complement fixation tests with immune sera made by injections with commercial egg lecithin Merck. With the flocculation method two of the preparations reacted only faintly. Also the brain lecithin and hydrolecithin gave no distinct reactions.

The immunization experiments of Sachs and Klopstock could easily be confirmed when commercial egg lecithin Merck was used for the injections. Immunizations with brain lecithin and hydrolecithin yielded no active sera. With an egg lecithin (No. 1) prepared by us the results were not satisfactory though a great number of injections was made. Only one serum gave reactions of medium strength by complement fixation and in flocculation tests with emulsions of Merck lecithin. It did not react however with the lecithin preparation No. 1 itself. In this respect the results resemble to a certain degree those of Sachs and Klopstock with their lecithin Böhringer immune serum. While the reactions of Merck lecithin were slightly diminished by the removal of cholesterol, addition of cholesterol to the lecithin No. 1 had no marked effect on the complement fixation tests, even when a larger amount was added than that present in the Merck preparation. The cholesterol content of this lecithin therefore does not suffice to account for the difference in the results. It is noteworthy that our lecithin immune serum No. 967 gave complement fixation with emulsions of cholesterol although this substance was not present in the injected material.

There are several plausible explanations for our results. According to one, the production of antibodies for lecithin would depend on certain physicochemical conditions of the emulsion injected or upon the presence of auxiliary substances in the lecithin preparation (*cf.* Sachs and Klopstock). Another possibility is that the active agent inducing the formation of antibodies is not lecithin itself but some other substance present in the active lecithin preparations. With regard to the latter assumption it may be mentioned that we obtained definite immunization effects from several injections of quantities

as little as 0.2 mg. of purified preparations of Forssman's heterogenetic haptene mixed with pig serum.[2]

To decide between the alternative explanations, further studies are necessary.

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[2] It may be stated that while in these experiments small quantities (1 mg.) of certain purified preparations of Forssman's heterogenetic haptene were active, no or a very slight effect was obtained on using larger amounts such as 100 mg. for each injection. These experiments will be fully described in a later communication.

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